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THE UNIVERSITY OF ALBERTA

A STUDY OF THE BACTERIA IN
RAW BULK TANK MILK

by

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A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES
IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE
OF DOCTOR OF PHILOSOPHY

DEPARTMENT OF DAIRY SCIENCE

EDMONTON, ALBERTA

SEPTEMBER 1963

ABSTRACT

In the spring of 1961 co-operative experiments were started at three centres in Canada to assess the value of preliminary incubation (P.I.) (55°F, 12.8°C for 18 hr) of milk samples before carrying out various bacteriological tests. The average increase of the standard plate count (S.P.C.) following P.I. was different at the three centres. In an attempt to explain these differences a study of the flora of milk samples before and after P.I. was undertaken. The results of this study suggested that the differences could not be attributed to a difference in the relative proportion of various types of micro-organisms in the raw milk (micrococci, streptococci, Gram-negative rods, etc.). It was deduced that the differences arose from the incidence of micro-organisms able to multiply in milk at the temperature of P.I.

The results on the effects of P.I. on the dye reduction tests indicated that P.I. reactivates micro-organisms which have been subjected to prolonged periods of refrigeration. This was evidenced by a greater correlation coefficient between resazurin reduction time and S.P.C. after, rather than before, P.I. There would appear to be a limited value in the use of the increase in S.P.C. following P.I. as a means of assessing the sanitary conditions under which milk is produced. Flora studies indicated that an increase in S.P.C. following P.I. merely reflected the presence of certain micro-organisms with the ability to multiply at 55°F (12.8°C). Thus contaminants which do not multiply at this temperature are neglected.

Further work on the origin of bacteria in milk, and their growth requirements would appear to be desirable.

An analysis of the flora of raw milk showed that in general the percentage of micrococci decreases, and the percentage of Gram-negative rods

increases with increasing colony count of the milk. In spite of these general trends marked variations were observed. The overall effect of P.I. on the flora of raw milk was to cause an increase in the percentage of Gram negative rods and a decrease in the percentage of micrococci. The effect on the percentage of streptococci was variable.

Studies on the flora of milking units showed that this was of variable composition and that it was affected by the type of sanitizing agent used.

Observations on the effect of different types of micro-organisms on the reduction of resazurin indicated that coliforms and streptococci, micrococci, and Gram-negative rods varied in their ability to reduce the dye. The micro-organisms are listed above in order of decreasing activity.

The pulsating rinse technique was used for the assessment of the bacteriological condition of milking units. The results of the standard plate count/milking unit showed little correlation with the standard plate count of the milk. It is suggested that clumps of bacteria in the rinse solution which subsequently broke down in the milk may have been responsible for the lack of correlation.

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GENERAL INTRODUCTION

INACTIVATION OF MICRO-ORGANISMS IN MILK BY COLD

The farm bulk milk cooler is now widely used for the storage of milk on farms before shipment to the local dairy. Bulk coolers maintain the milk at temperatures of 34 - 40°F (1.1 - 4.4°C) which practically eliminates the multiplication of bacteria present in the milk. As a result of this efficient refrigeration it is now possible for the milk producer to be less fastidious in cleaning and sanitizing the milking equipment and still satisfy the required bacteriological standards. This is less than desirable. Efficient cooling has the effect of inactivating bacteria present in the milk and as a result, milk having a high bacterial count may give satisfactory results when subjected to either the methylene blue or resazurin reduction tests. This is because the rate of reduction of the dye in both tests is dependent on the metabolic activity of the micro-organisms.

It has been demonstrated by several workers that the holding of milk samples at low temperatures will adversely affect the activity of bacteria in milk, resulting in an increase in the time of dye reduction tests. Ellenberger and Moody (1930) found an increase in methylene blue reduction time of 15 min if the milk samples were placed in iced water for 2 hr before the test. An increase of 5% in the reduction time of methylene blue following milk storage at 40°F (4.4°C) for 24 hr was noted by Frayer (1934). This worker (1938) also found a similar effect with the resazurin test. Powell et al. (1939) stored milk samples at 40°F (4.4°C) overnight and found a methylene blue reduction time 3½ hr greater than with duplicate samples stored at 60°F (15.5°C). Wilson et al. (1935) reported that the

icing of milk samples increased the methylene blue reduction time. Smythe (1945) showed by experiments that prolonging the storage of milk samples at 5°C resulted in a progressive increase in methylene blue reduction time. This worker also showed that storage temperatures above 10°C resulted in decreased reduction times. Morton and Vincent (1949) reported that the storage of milk samples for 24 hr in iced water affected the resazurin reduction time but had little effect on the methylene blue reduction time. Eddison et al. (1951) showed that storage of milk samples at a temperature of 3.6°C for 2 hr markedly increased the methylene blue reduction time, when compared with duplicate samples stored at 10°C for 22 hr.

The work of Thomas and Davies (1940) indicated little decrease in mean resazurin reduction times following refrigerated storage. Thomas (1941) found that the storage of milk at either 5 or 15°C for 24 hr caused a considerable increase in the resazurin reduction time, the increase being the same for both of the temperatures mentioned. The results at 15°C are surprising as bacterial growth would be expected at this temperature with a resultant decrease in the reduction time. Thomas et al. (1946) modified the preliminary incubation to 16°C for 24 hr when using the resazurin test. Johns (1952) found that the grade assigned to milk samples, as measured by dye reduction tests, was improved by storage in iced water before testing; however, none of the differences was statistically significant.

With resazurin reduction tests, it is difficult to distinguish between the effect of refrigeration on the bacteria and leucocytes. Revallier-Warffemius (1947) and Galesloot (1948) considered that increases in resazurin reduction time as a result of refrigeration were caused by a decrease in the activity of leucocytes. This result however is probably a combined effect of refrigeration on both bacteria and leucocytes. Morris (1944) found that leucocyte activity, as measured by resazurin reduction, varied with the storage temperature as illustrated in Table 1.

TABLE 1

Variation in the rate of reduction of resazurin due
to holding the milk at different temperatures
(After Morris, 1944)

	<u>Resazurin Disk reading at the end of</u>		Complete reduction of resazurin
	10 min	1 hr	
Tested immediately after production	4	0	1 hr
Tested after holding at 55°F overnight	4½	1½	1½ hr
Tested after holding at 40°F overnight	0	0	10 min
Tested after holding at 32°F overnight	2½	1	1½ hr

The reason given by Morris for the inconsistencies in resazurin reduction as a result of lowering the storage temperature of the milk (shown in Table 1) was that the metabolism of leucocytes in milk is catabolic, hence the lower the temperature of storage (outside the limits of physiological damage) the longer should be the life and activity of the leucocytes.

As cold storage of milk, resulting in inactivation of micro-organisms present, prevents the dye reduction tests from detecting milk of poor bacteriological quality, Johns (1930) proposed a preliminary incubation (P.I.) of the milk samples at 55°F (12.8°C) for 18 hr before testing by the methylene blue test. This modification was found superior for determining the bacteriological quality of milk in comparison with unincubated milk as measured by the methylene blue, standard plate count, direct microscopic and acidity tests.

The value of storage before testing milk with the methylene blue test was also noted by Wilson et al. (1935). As a result of their studies the holding of milk samples at atmospheric shade temperature overnight (12 - 18 hr) became statutory for the methylene blue test on designated milk in England and Wales in 1937.

REACTIVATION OF MICRO-ORGANISMS IN MILK BY PRELIMINARY INCUBATION

The observation of Thomas and Tudor (1937) that high count milks could escape detection by the methylene blue test during the winter months, when the atmospheric shade temperature was low, led Powell et al. (1939) to recommend that milk samples be held overnight at 50 - 59°F (10 - 15°C) before testing. Chalmers (1938) reported that the relationship between methylene blue reduction time and keeping quality, as determined by taste, was much better if the milk were incubated for 15 - 24 hr at 15°C than if the tests were carried out on fresh milk. Thomas et al. (1946) after carrying out investigations on the resazurin test suggested P.I. at 16°C for 24 hr. Further research by Johns (1958) led to the suggestion that P.I. of 18 hr at 55°F (12.8°C) be introduced as an aid to milk quality control.

Hadland (1960) stated that P.I. at 15 - 17°C for 24 hr before the methylene blue test gave a better indication of the bacteriological quality than the same test without P.I. The conditions of incubation were varied in further experiments (Hadland, 1962a; Hadland and Bo, 1962) and a recommendation made for P.I. at 12°C for 24 hr. Earlier results obtained by Scandinavian workers, Berger and Anderson (1949), Bertelsen, Mattsson and Dufeu (1956) and Olson (1956) stressed the value of P.I. at 17°C for 24 hr before testing.

Chalmers (1956) suggested that P.I. at 60°F (15.5°C) for 21 hr

would reflect the care taken in milk production, because the saprophytic flora derived from dirty utensils, etc. would multiply during such incubation whereas micro-organisms from the udder would not. This aspect was further discussed by Johns (1960) who pointed out that efficient refrigeration provided by bulk milk coolers interferes with the assessment of conditions of production of milk and bacteriological quality of the milk for the following reasons:

(1) Because of the low temperature of milk storage, unsatisfactory conditions of production may prevail on the farm with little effect on the quality of the milk because bacterial growth in the bulk tank is virtually eliminated.

(2) The milk is collected from the farm every 48 hr, during which time the milk is held at 34 - 40°F (1.1 - 4.4°C) which inactivates the bacteria present in the milk.

P.I. as suggested by Johns (1958), 18 hr at 55°F (12.8°C) might be expected to provide more valuable information on the bacteriological quality of raw milk by overcoming the two factors mentioned above. In the first place the bacteria would regain their normal metabolic activities and as a result, would be more active at the start of the dye reduction tests. In the second place any multiplication of micro-organisms might be useful as an indication of the conditions under which the milk was produced. For example, if the bacterial flora of the milk as it leaves the udder is almost wholly composed of udder micrococci, with little ability to multiply at 55°F (12.8°C) (Gibson and Abd-el Malek, 1957), such organisms would not grow during P.I. and any growth that did occur must be attributed to saprophytes present in the original milk. These saprophytic micro-organisms must be regarded as being indicative of poor

methods of production. In connection with this point Pette (1962) considered that while P.I. may reflect the sanitary condition of the equipment it does not show up other factors of hygienic milk production, such as poor housing conditions for the cows, etc. However, Hadland (1962b) considered this aspect adequate for the practical test. Baskett (1962) and others recommended that "where milk has been cooled at very low temperatures for long periods it is desirable to pre-incubate samples before carrying out tests".

As a result of the favourable reactions to P.I. in many countries, Dr. C.K. Johns, Canada Department of Agriculture, initiated co-operative experiments to try and design a bacteriological test for milk which would indicate sanitary conditions of production even when the milk was stored cold in a bulk tank. These experiments were carried out at the University of Manitoba (Dr. J. Nesbitt), Ontario Agricultural College, (Professor A. Leggatt) and the University of Alberta (Dr. L.F.L. Clegg). Some of the results of these experiments are considered in Part I of this thesis. In the course of these experiments it soon became evident (Johns, 1961) that the results obtained at the three centres were different, especially with respect to the standard plate counts before and after P.I. It appeared that the most probable explanation for these differences would be found by studying the flora of the milk and the milking equipment. For this reason, a study of this aspect was undertaken; the results of which are reported in Part II of this thesis.

PART I

THE EFFECT OF P.I. ON THE STANDARD PLATE COUNT AND THE RESAZURIN REDUCTION TEST AND SOME OBSERVATIONS ON THE PULSATING RINSE TECHNIQUE

METHODS

As mentioned previously experiments were started in 1961 at three centres in Canada to assess the value of the various tests used to estimate the bacteriological quality of raw milk, with special reference to P.I.

The first step was to select twenty farms with standard plate counts of $< 50,000/\text{ml}$, farms with a plate count $> 50,000/\text{ml}$, being considered of poor quality and which would be detected without P.I. The selection of farms was done by making standard plate count tests on all producers shipping milk to Silverwood's Dairies Limited, Edmonton. After this initial screening the farms were checked for type of equipment (absence of pipelines), number of milking units (preferably 3 or 4), and the willingness of the owners to co-operate.

At each farm visit a farm report sheet was completed giving details of the conditions under which the milk had been produced - e.g. physical condition and cleanliness of milking equipment, types of detergent and sanitizer used, method of care of rubber parts, etc.

Each of the twenty farms selected was visited at approximately monthly intervals, at which time a sample of the milk was collected and a pulsating rinse (Claydon, 1953) made of the milking units. The milk at the time of sampling was made up from two milkings.

SAMPLING

Pulsating Rinses

Long tube bucket units. The milking unit was assembled and sanitized according to the procedure used before milking. The cluster was then set up on a specially constructed stand with the teatcups held upright as described by Johns and McClure (1961). The valve on the milk line was closed and sterile buffered rinse solution (containing suitable disinfectant neutralizers, Standard Methods p 265) was poured through one teatcup until it came within an inch of the top. The unit was then connected to the vacuum line and allowed to pulsate for 1 min. After this the valve on the milk line was opened, allowing the rinse solution to be sucked into the milking bucket. The rinse solution was swirled around inside the bucket to bring the solution in contact with the entire inner surface. The rinse solution was then poured back into its original container. This procedure was repeated on the other milking units.

Suspended bucket units. The milking unit was assembled and sanitized according to the usual procedure and set up in the special stand described above. A clamp was then placed on the milk tube of each inflation and the teatcups were filled with sterile buffered rinse solution to within an inch of the top. The unit was then connected to the vacuum line and allowed to pulsate for 1 min. The clamps were removed allowing the rinse solution to be drawn into the milking bucket. The procedure was then the same as for the long tube bucket units.

Milk

The milk to be sampled from the bulk tank cooler was agitated for 5 min. A sterile dipper was used to obtain the sample which was transferred into a sterile plastic sample bag (Nasco Inc., Fort Atkinson, Wis.).

TESTING OF SAMPLES

At the laboratory the rinse solutions were tested immediately and each milk sample was divided into several sub-samples, one set being kept in the cooler overnight (39°F) (3.9°C), the other set being incubated at 55°F (12.8°C) for 18 hr.

The rinse solutions were tested by the standard plate count (Difco agar) (Standard Methods p. 47) with incubation at 32°C for 2 days (plates were poured in duplicate). Other tests were also made; i.e. psychrophile count, coliform count and the laboratory pasteurized count, but the results of these tests are not reported here.

The milk samples both fresh (which had been cold-stored overnight) and P.I. were tested the next day. The fresh milks were tested by the standard plate count (32°C for 2 days), and by the resazurin reduction test at 35°C. In addition the following tests were done but the results are not reported here: psychrophile count, coliform count, laboratory pasteurized count, direct microscopic count (both for bacteria and leucocytes) and a test for the presence of antibiotics.

For the P.I. milk the above tests were done with the exception of the laboratory pasteurized count, the leucocyte count and the antibiotic test.

RESULTS

The complete results of the co-operative experiment carried out at the three different centres in Canada are not presented here in full. The reasons for this are that the author did not initiate or plan the original experiment and the complete results from the three centres are undergoing computer analysis in Ottawa and the final results will probably

be published in the near future (Johns, 1963). However, the more pertinent results obtained by the author are reported as a basis for the development of the work on the flora (Part II) and for reference in connection with the final discussion and conclusions.

The results reported here are: (1) The relationship between the standard plate count and the resazurin reduction time, both before and after P.I. of the milk samples. (2) The relationship between the standard plate counts before and after P.I. of the milk samples. (3) The pulsating rinse as a means of assessing the bacteriological state of the milking equipment.

THE RELATIONSHIP BETWEEN THE STANDARD PLATE
COUNT AND THE RESAZURIN REDUCTION TIME,
BEFORE AND AFTER PRELIMINARY INCUBATION

The data obtained at this centre between the months of June, 1961 and July, 1962, are illustrated in Figs. 1 and 2. Five samples with reduction times 3 hr were, for convenience, omitted from the graphs, but have been included in the statistical calculations.

The results indicate a higher correlation between the standard plate count (S.P.C.) and resazurin reduction time (R.R.T.) after P.I. ($r = -0.75$) than between the S.P.C. and R.R.T. before P.I. ($r = -0.69$). This is in accord with the findings of Johns (1960). It is not possible however to say with certainty whether this difference is real or inherent in the experimental procedure. On arrival at the laboratory the fresh milk samples were kept in the cooler for 18 hr at 39°F (3.9°C), whereas the P.I. samples were incubated immediately, in both cases the dye being added to the milk. Thus at the beginning of the resazurin reduction test at 35°C the next day the fresh milk had in fact been subjected to an additional period of inactivation, although Johns (1952) indicated that this would make little difference

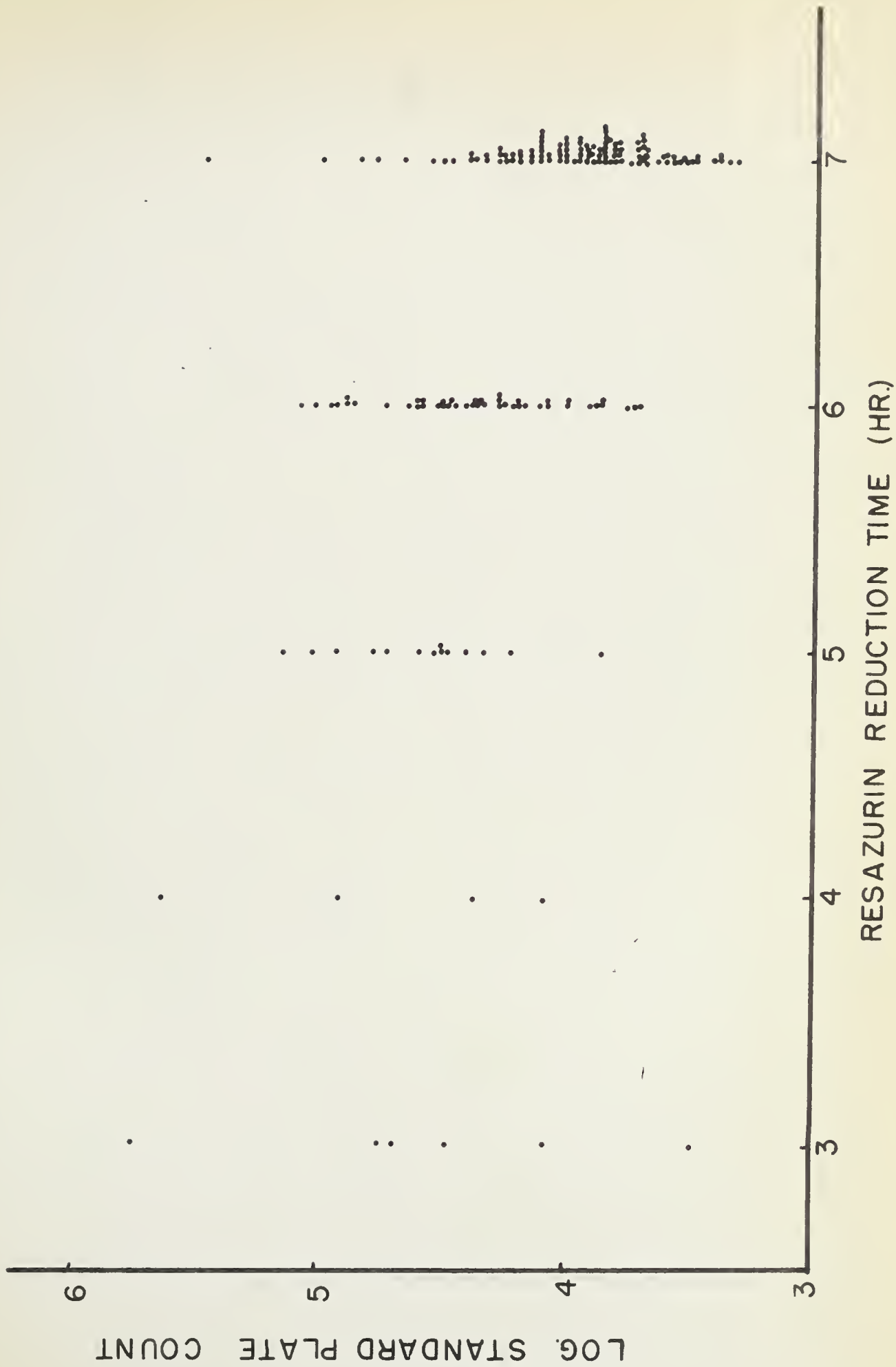


FIGURE 1. The relationship between standard plate count and resazurin reduction time on fresh milk

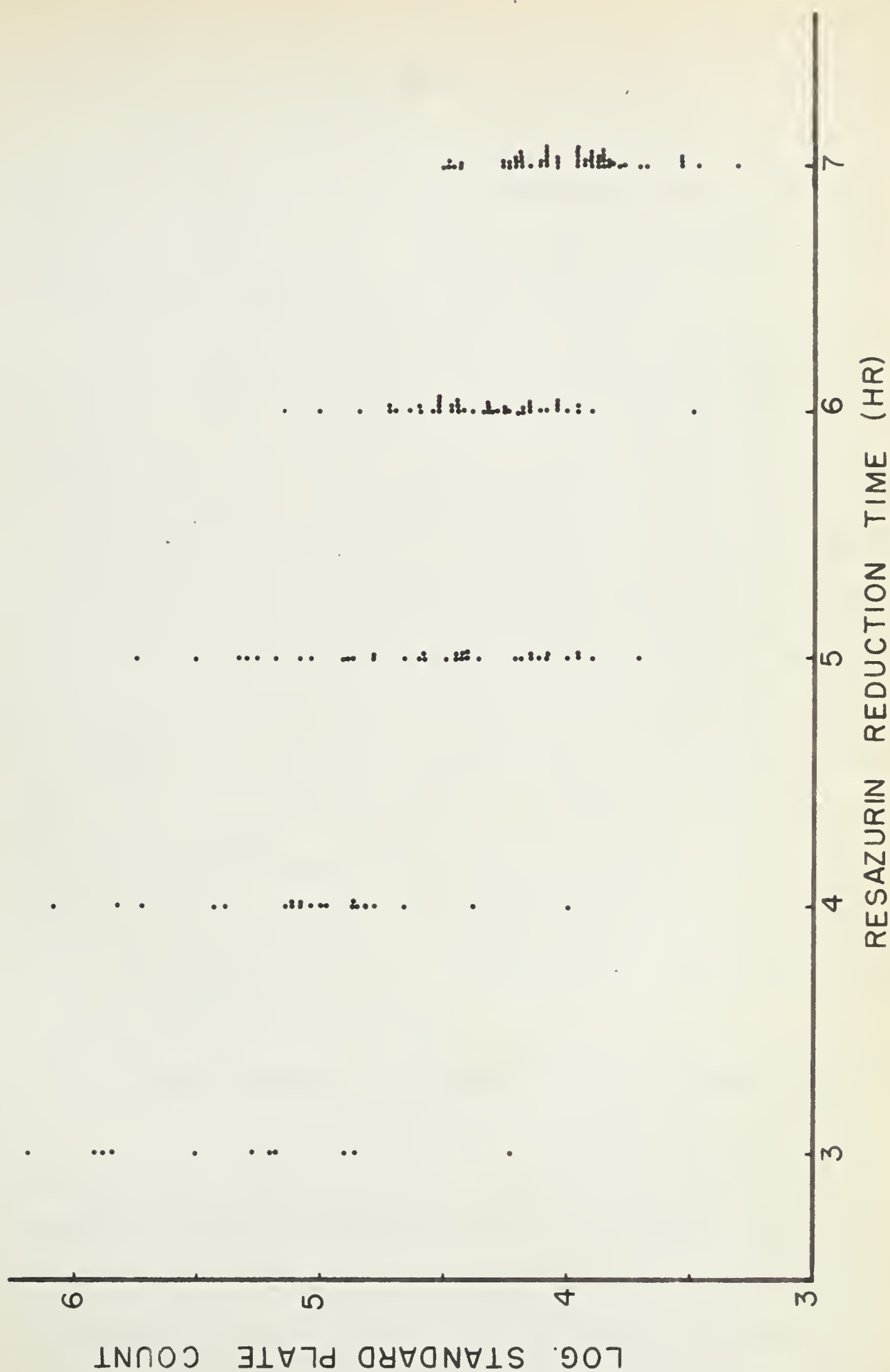


FIGURE 2. The relationship between standard plate count and resazurin reduction time on milk after P.I.

to the results of the test. However, it is felt that immediate testing of the fresh milk on arrival at the laboratory would have eliminated the possibility of further inactivation as a result of overnight refrigeration.

Reference to the results of Morris (1944) shows that the day after sampling the leucocytes in the fresh milk would be more active than in the milk stored at 55°F (12.8°C). There is little doubt that the effect on the leucocytes would affect the correlation coefficients.

A final reason why the correlation coefficients may not be so different as they appear is that the data used for computing these values are somewhat skewed because after 6 hr the resazurin reduction test was terminated, and any milks not having reached the Munsell 5P7/4 end-point were recorded as having reduction times of 7 hr. As would be expected the number of samples of fresh milk with reduction times of 7 hr was greater than for the samples of P.I. milk because of the lower standard plate counts of the fresh milks. As a result the fresh milk data include 107 samples with reduction times of 7 hr whereas data from the P.I. milks include only 58 samples with reduction times of 7 hr. As a reduction time of 7 hr means an indefinite time greater than 6 hr it is not possible to determine the effect of this skewing of the data on the final correlation coefficients. Only by observing the reduction test of each sample to the 5P7/4 end-point would it be possible to determine the exact correlation coefficients.

The relationship between the standard plate count before and after P.I.

It soon became obvious (personal communication, Johns, 1961) that the results from the three centres, as well as results obtained in Ottawa, differed in respect to the average increase in standard plate count following P.I. This is illustrated in Figs. 3-6. The data from Ottawa and Winnipeg have a wider spread than the data from Guelph and Edmonton. In an attempt to explain these differences investigations were started at the University of Alberta into the bacterial flora of milk and milking equipment (see Part II).



FIGURE 3. The relationship between the standard plate count on milk before and after preliminary incubation at 55°F (12.8°C) for 18 hr.

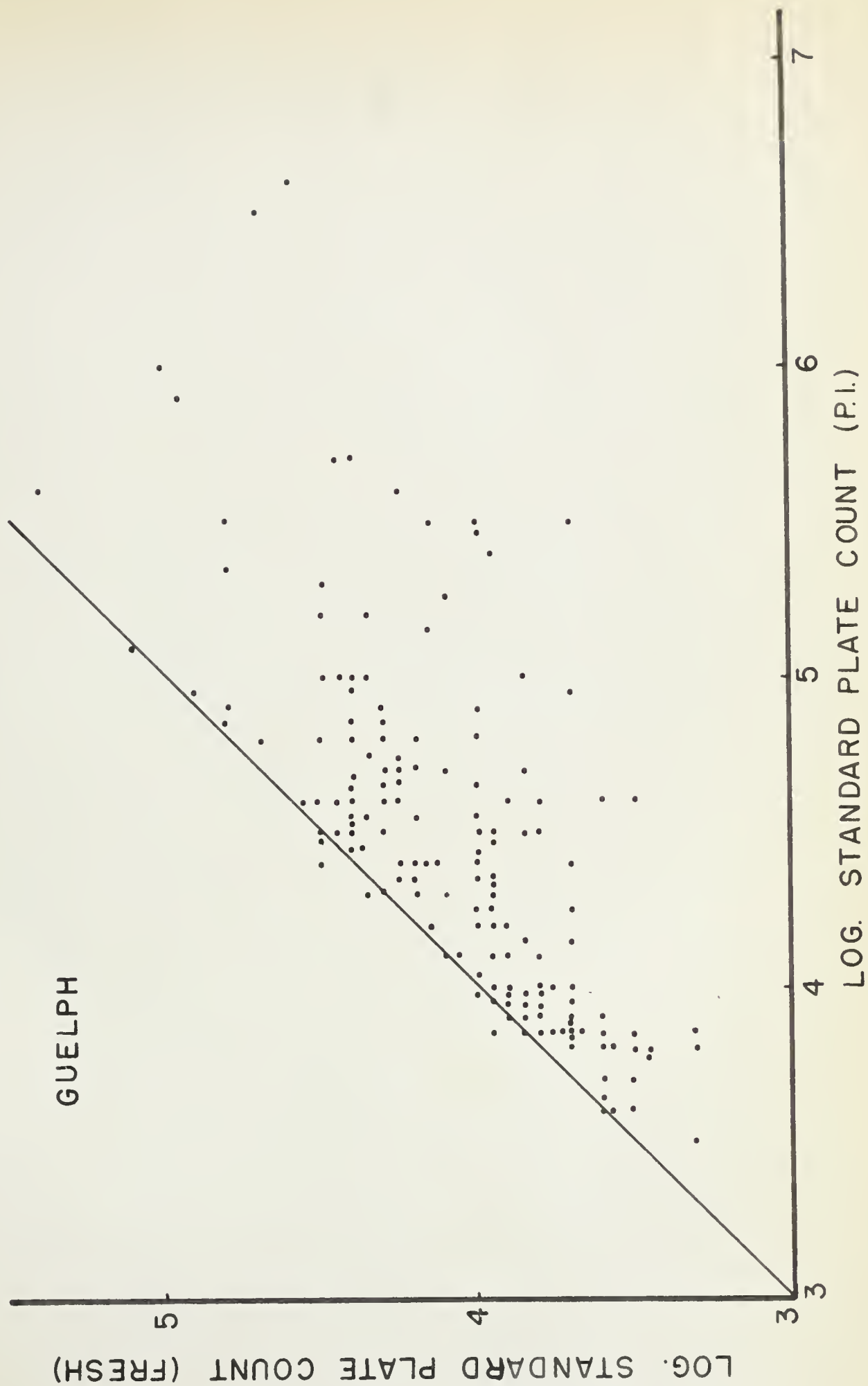


FIGURE 4. The relationship between the standard plate count on milk before and after preliminary incubation at 55°F (12.8°C) for 18 hr.

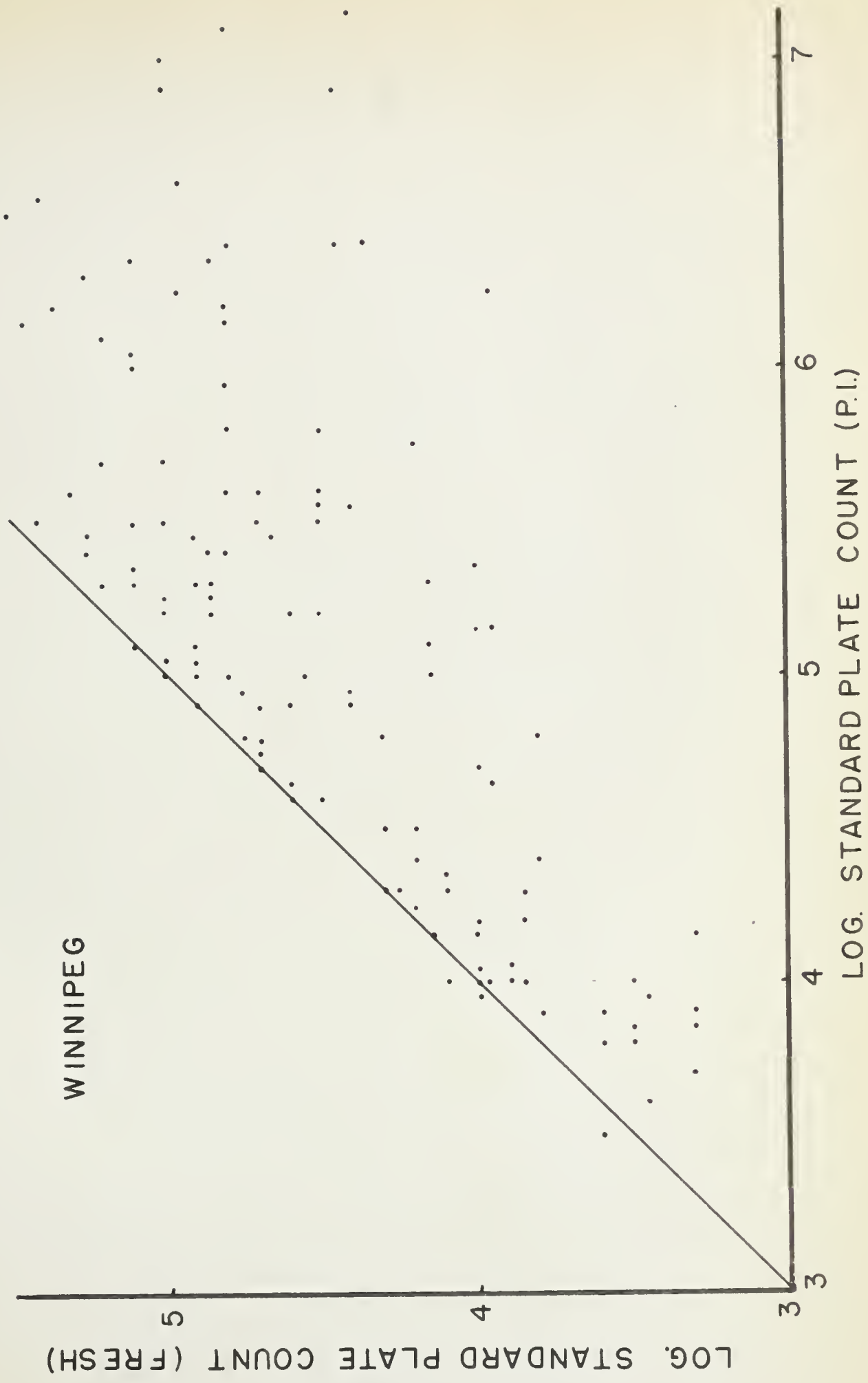


FIGURE 5. The relationship between the standard plate count on milk before and after preliminary incubation at 55°F (12.8°C) for 18 hr.

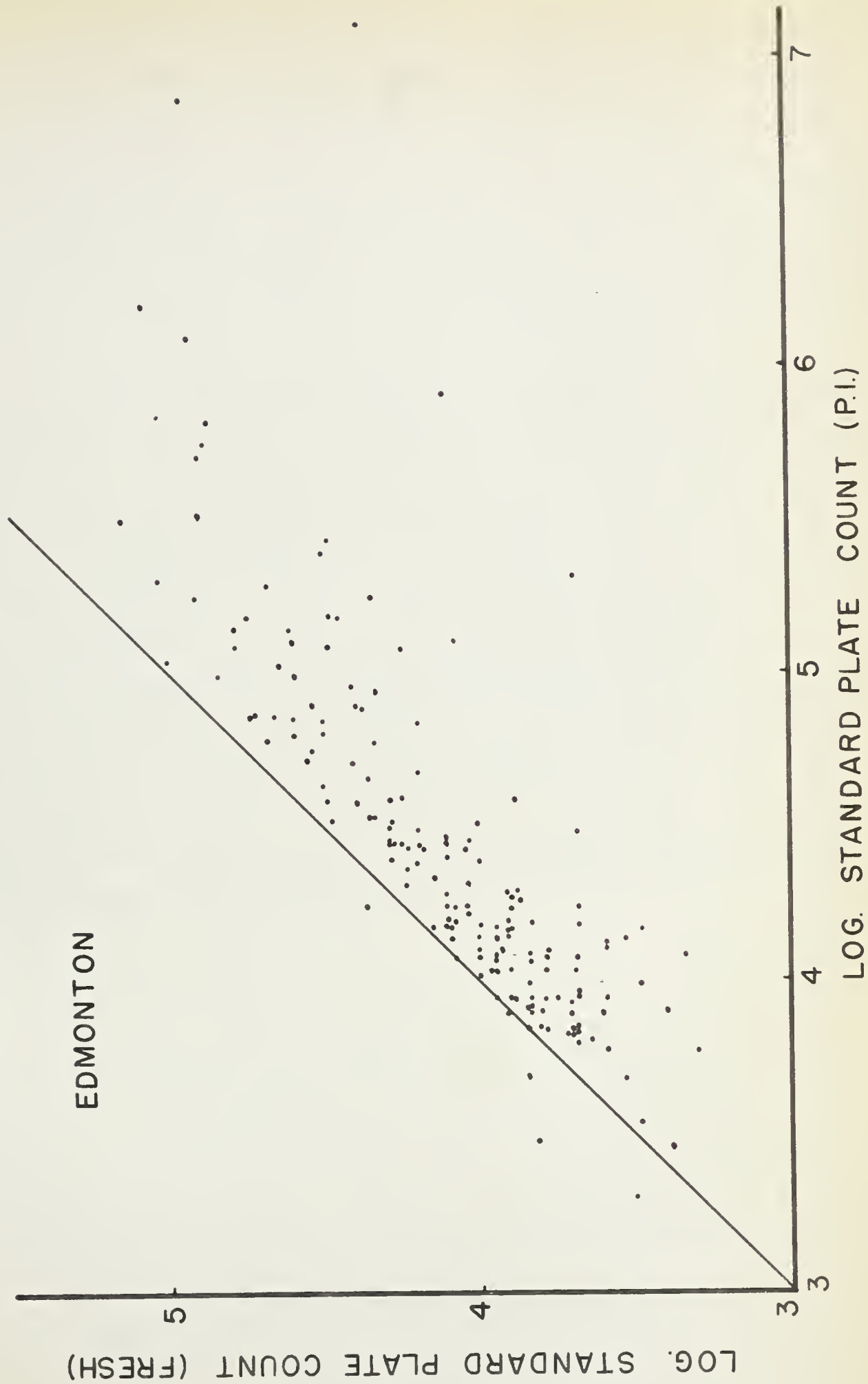


FIGURE 6. The relationship between the standard plate count on milk before and after preliminary incubation at 55°F (12.8°C) for 18 hr.

Pulsating Rinses

The method described earlier is a modification of the technique of Claydon (1953) who reported that pulsating rinses gave much higher plate counts than static rinses on the same equipment. This would be expected because of the pulsating action of the inflations. It was anticipated that the pulsating rinse count would give a good indication of the bacteriological state of the milking equipment and be closely related to the standard plate count of the milk. The results are given in Figure 7.

Without statistical analysis it is obvious that the pulsating rinse count shows little relationship with the standard plate count of the milk. Because of the great variability of the results one must question the value of the pulsating rinse technique. The only conclusion that can be made from the data that may be of any value is that in 85% of the milk samples with a plate count $> 50,000/\text{ml}$ the pulsating rinse count was $> 1,000,000/\text{milking unit}$. Unfortunately the reverse argument would not appear to hold i.e. that a pulsating rinse count of $> 1,000,000/\text{milking unit}$ indicated a plate count on the milk of $> 50,000/\text{ml}$. These results might suggest that milk of $> 50,000$ bacteria/ml be regarded as being produced under unsanitary conditions.

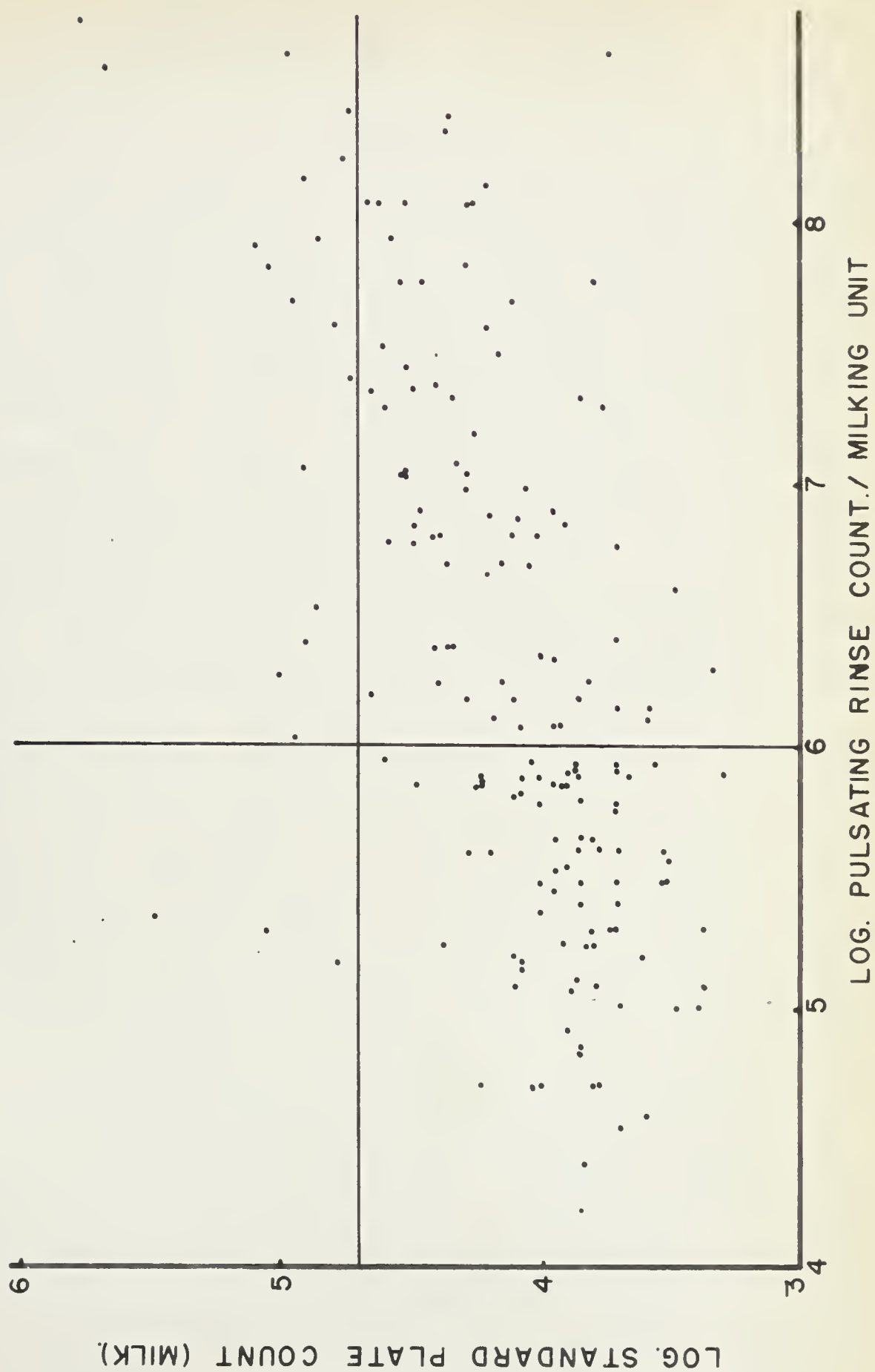


FIGURE 7. The relationship between the standard plate count of milk and the pulsating rinse count of milking units.

P A R T I I

STUDIES ON THE BACTERIAL FLORA OF RAW
BULK TANK MILK AND OF MILKING EQUIPMENT

INTRODUCTION

As mentioned in the General Introduction the prime reason for undertaking studies on the bacterial flora of raw bulk tank milk was to obtain information that might help to explain differences in results obtained at Ottawa, Winnipeg, Guelph and Edmonton, However, this is not the only reason why a study of the flora of raw milk is desirable. When the tests used to evaluate the bacteriological quality of raw milk are examined it can be seen that in many cases the different tests are carried out at different incubation temperatures, i.e. the methylene blue reduction test at 35 - 37°C, the resazurin reduction test at 35 - 37°C, the standard plate count at 32 or 35°C and various keeping quality tests at 16 - 26°C. Preliminary incubation (P.I.) as suggested by Johns (1958) introduces yet another temperature to the list. If one now considers that the bacterial flora of raw milk is composed of many different types of micro-organisms, varying in their optimal growth temperatures, it can be seen that the various tests are in fact weighted in favor of different types of micro-organisms. Only with a thorough knowledge of the flora of raw milk can this weighting be evaluated. The components of the flora play different roles at different temperatures and this is well illustrated by the results of Garvie and Rowlands (1952a,b). These workers carried out dye reduction tests at 37.5°C and a clot-on-boiling test at 22°C. It was found that staphylococci were the dominant group of micro-organisms at the end of the dye reduction tests, whereas these

micro-organisms constituted only a small proportion of the flora at the end-point of the clot-on-boiling test. Thus the two tests, as a result of the different incubation temperatures, would appear to be indications of two different sets of conditions. Wilson et al. (1935), Hobbs (1939), Thomé (1941) and Jones and Davies (1944) all noted the effects of different bacterial types on dye reduction tests.

Other reasons why a knowledge of the flora of raw milk is desirable are that the quality of products made from the raw milk will be affected by the types of bacteria present, e.g. Galesloot (1956). In addition a knowledge of the incidence of pathogenic bacteria has considerable significance to public health authorities. A further reason is that the flora of raw milk should be investigated and reported upon as a matter of academic interest.

In view of the reasons put forward above, it would be expected that a survey of the literature would reveal a considerable number of papers on the flora of raw milk. Surprisingly, this is not the case, the majority of relevant papers being primarily concerned with some other aspect. Thus there have been many papers published on incidence of pathogenic micro-organisms, the coliform group, the streptococci, the lactobacilli, etc. in raw milk, but very few report the results of a complete survey of the bacterial types present.

There is general agreement that when milk leaves the udder of a healthy cow the flora consists mainly of micrococci together with smaller numbers of staphylococci and corynebacteria; see Harding and Wilson (1913), Evans (1916), Copeland and Olson (1926), Dorner (1930), Thornton and Strynadka (1935), Johns (1936), Gibson and Abd-el Malek (1940 and 1957), Nakanishi and Hyogo (1962). Other data on the flora of raw milk, not aseptically drawn are reported here.

Gibson and Abd-el Malek (1940) while studying the effect of low temperature pasteurization made some observations on the flora of raw, Certified and bulked milk; these are given in Table 2.

TABLE 2

A comparison of the flora of Certified and bulked milk
(from Gibson and Abd-el Malek 1940)

Date	Sample No.	Standard plate count (/ml)	Percentage of bacterial types:			
			Strepto-cocci	Micro-cocci	Coryne-bacteria	Others
CERTIFIED MILK						
Dec.	1	9400	56.5	41.0	2.5	-
Mar.	2	2900	21.4	60.7	17.9	-
Apr.	3	11000	6.4	19.1	49.0	25.5
Apr.	4	17000	9.0	45.5	18.2	27.3
Sep.	5	4450	17.5	82.5	-	-
Oct.	6	17000	2.5	60.0	32.5	5.0
BULKED MILK						
Dec.	1	345000	44.8	27.6	17.2	10.4
Feb.	2	870000	63.6	3.0	6.1	27.3
Mar.	3	3650000	62.9	25.7	-	11.4
Apr.	4	920000	81.5	3.5	-	15.0
Nov.	5	1130000	60.5	11.6	2.3	25.6

These results indicate that milk, when produced under clean conditions, has a dominant flora of micrococci.

McKenzie and Bowie (1946) studied the visual conditions on farms as indicators of the keeping quality of milk. It was found that 'visually good' farms could have poor milk, and 'visually poor' farms have good milk. The results showed that different flora prevailed under the different conditions. This is illustrated in Table 3.

TABLE 3

The bacterial flora of milk produced on farms classified as 'poor' and 'good' on visual examination
(from McKenzie and Bowie, 1946)

	Total Colonies	Micro- cocci	Strepto- cocci	Gram + ve rods	Gram - ve rods	
					Acid	Alkali
<u>a. 'Poor' Farm</u>						
Milk	78	26 (33%)	9 (12%)	9 (12%)	3 (4%)	31 (39%)
Equipment	143	70 (49%)	16 (11%)	7 (5%)	4 (3%)	46 (32%)
<u>b. 'Good' Farm</u>						
Milk	61	14 (23%)	33 (54%)	4 (7%)	4 (7%)	6 (9%)
Equipment	202	89 (44%)	53 (26%)	20 (10%)	18 (9%)	22 (11%)

Further interesting results were found when they studied the flora of the milk and equipment under conditions of hand and machine milking (Table 4).

The results show that under different conditions of production different flora were apparent.

In an investigation on the effect of added hypochlorite on the numbers and kinds of bacteria in milk, McKenzie and Booker (1955) studied the initial flora. In 137 cultures they obtained the following percentages of types: Streptococci, 19%; Micrococci, 68.8%; Gram-negative rods, 3%; Gram-positive rods, 9.4%.

TABLE 4

The flora of milk produced under conditions of hand and machine milking on a 'visually good' farm (from McKenzie and Bowie, 1946)

	Total Colonies	Micro-cocci	Strepto-cocci	Gram + ve rods	Gram - ve rods	
					Acid	Alkali
<u>Hand Milking</u>						
Milk	15	3 (20%)	12 (80%)	0	0	0
Equipment	22	1 (5%)	15 (68%)	2 (9%)	0	4 (18%)
<u>Machine Milking</u>						
Milk	15	6 (40%)	2 (13%)	2 (13%)	5 (34%)	0
Equipment	45	25 (55%)	13 (29%)	4 (9%)	3 (7%)	0

Carreira et al. (1955) (Table 5) also gave data for the flora of raw milk.

TABLE 5

Distribution of bacterial types in fresh milk (from Carreira et al. 1955)

Group*	Micrococci	Streptococci	Gram + ve rods	Gram - ve rods
1	90.0	2.1	4.5	2.1
2	97.2	0.0	0.0	1.1
3	72.8	0.0	2.0	22.2
4	75.3	11.1	1.2	4.9
5	69.1	5.8	22.3	0.7
6	37.7	49.8	5.4	7.0
7	78.5	7.6	3.2	7.5
8	31.8	43.9	1.2	21.9

The above results are given as percentages

*Each group is an average of 32 samples

The most recent and comprehensive work on the flora of raw milk is that of Thomas et al. (1962). These workers isolated and identified 2065 bacterial cultures from 87 farm milk supplies. In order to avoid repetition the relevant data of Thomas et al. will not be given here but will be included in the section devoted to results and discussion. Thomas et al. (1963) also published data on the flora of farm dairy equipment and the results of this work will be referred to later.

The results of the present investigation of the flora of raw milk and milking equipment may be divided into four sections as follows:

- Section 1. Preliminary studies on the flora of raw bulk tank milk, before and after P.I., and the flora of milking units.
- Section 2. The flora of raw bulk tank milk.
- Section 3. The flora of raw bulk tank milk before and after preliminary incubation with some observations on the resazurin reduction test.
- Section 4. The flora of raw bulk tank milk before and after preliminary incubation, and the flora of milking units.

Section 1

Preliminary Studies on the Flora of Raw Bulk Tank Milk Before and After Preliminary Incubation and the Flora of Milking Units

The results reported here were obtained during the period November 1961 - June 1962 in the course of the investigation into the value of P.I.

Methods

Twenty-three plates containing between 30 - 300 colonies derived from raw milk were selected over a period. From each of these plates 20 colonies were picked off into litmus milk (Difco). The colonies to be picked off were determined by the use of a 4 x 5 line grid. This was drawn on the back of the plates with a grease pencil to give a pattern of squares with 20 intersections. The 20 colonies nearest to the 20 intersections were the colonies selected. They were picked off using a platinum loop. After incubation at 32°C for 3 days the reactions of the bacteria in the litmus milk were recorded. The litmus milk cultures were then streaked onto plates of standard plate count agar and incubated for a further 24 hr at 32°C. After this period of incubation they were stained by the Gram-stain (Jensen's modification - see Mackie and McCartney, pp 112-113) and examined microscopically, the staining reaction and shape of the organisms were recorded. Following a catalase test on the plate cultures the micro-organisms were classified into 4 groups: Micrococci, Streptococci, Gram-negative rods and 'others'. A similar procedure was carried out on 18 samples of milk after P.I. and also on 14 pulsating rinses.

Results and Discussion

The purpose of these investigations was to obtain a general picture of the flora of raw milk, raw milk after P.I. and milking units. For this

reason the plate count of the samples was not recorded. The averaged results are shown in Table 6.

TABLE 6

Typing of colonies isolated from raw milk
before and after P.I. and from utensil rinses

Sample	Micro- cocci	Strepto- cocci	Gram - ve Rods	Others
Raw milk	56%	22%	4%	18%
Milk after P.I.	28%	25%	42%	5%
Rinses	40%	42%	9%	9%

The results of individual samples showed very marked variation. Thus the percentage micrococci in the fresh milk ranged from 15 - 87%, in the P.I. milk from 0 - 70%, and in the rinses from 20 - 100%. This wide variation suggested that the expression "the normal flora of milk", widely used in textbooks, had in fact very little meaning. In view of the above results it was decided to investigate the flora of raw milk and its relationship to the standard plate count, the results of which are reported in Section 2.

Section 2

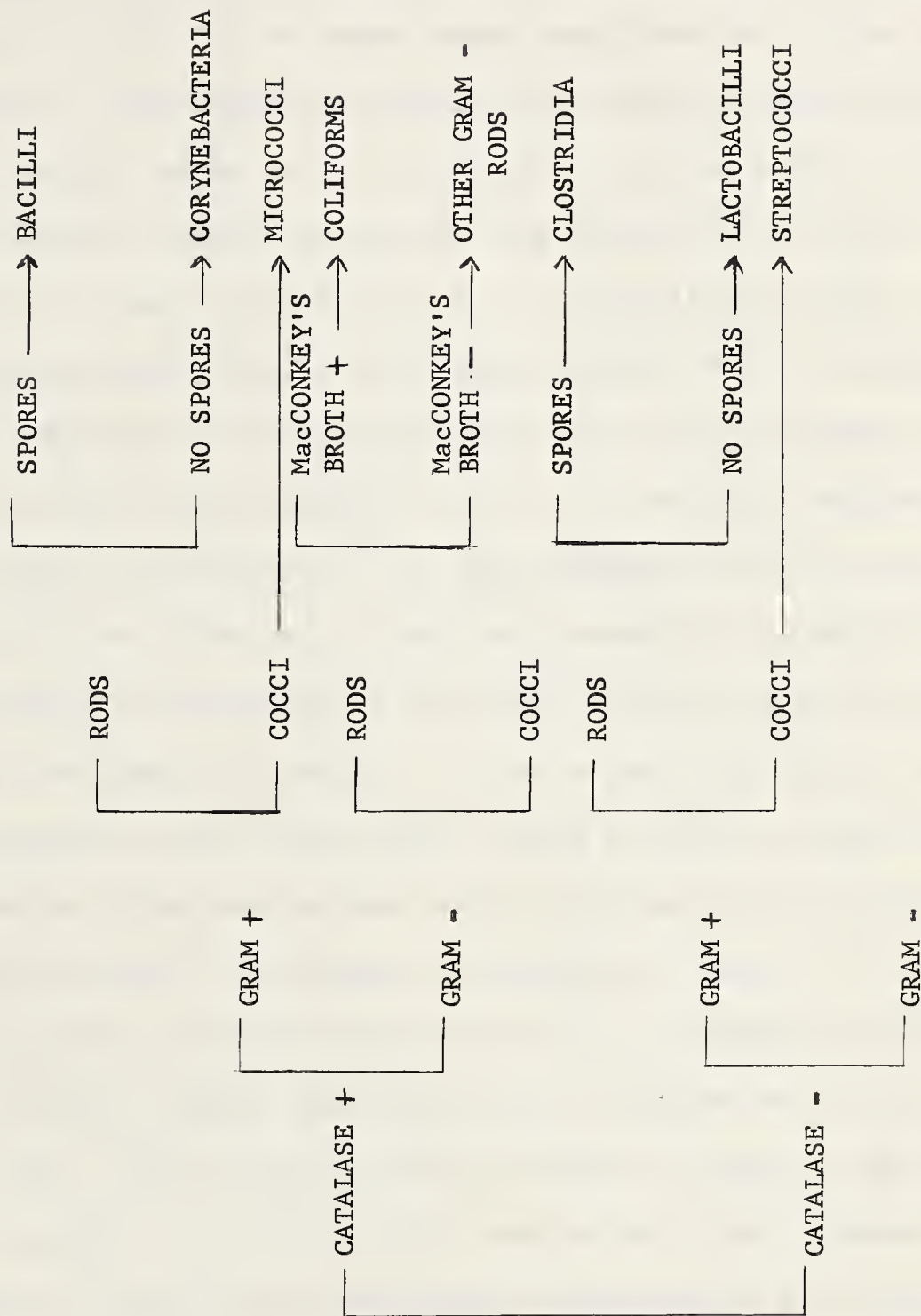
The Flora of Raw Bulk Tank Milk and the Relationship of the Flora to the Standard Plate Count

During December 1962 milk samples were collected from 141 different farms. The samples were picked up by milk truck drivers (Silverwood's Dairies Limited) when the milk in the bulk tanks were two days old.

Methods

The procedure for isolation and identification of the flora was slightly modified from that described in Section 1. For convenience the modified procedure is given here in fairly full detail.

The samples were plated on standard plate count agar on the day of collection and were incubated at 32°C for 48 hr. After this incubation the colonies were counted and plates (containing between 30 - 300 colonies) were chosen; from these 20 colonies were picked off into litmus milk using the grid previously described. After incubation at 32°C for 3 days in litmus milk the reaction was recorded and the cultures streaked onto poured plates of standard plate count agar and incubated for a further 24⁰hr at 32°C. Cultures from the plates were Gram-stained (Jensen's modification - see Mackie and McCartney, pp. 112-113) and examined for morphology. Any gram-negative rods were inoculated into MacConkey's broth and incubated at 37°C for 24 hr for the detection of coliforms, and any Gram-positive rods were put on agar slopes and incubated at 32°C to allow spore formation. Cultures from the slope were stained with crystal violet and examined for the presence of spores. Catalase production was detected by adding a drop of hydrogen peroxide solution (10% v/v) to the cultures on the agar plates. From the results of these tests it was possible to classify the isolates according to the scheme shown in Fig. 8. This scheme obviously does not



A FIGURE 8. Scheme for Identification of the Main Types of Bacteria

include all the different types of micro-organisms that are likely to be present in milk, nor does it classify all types to the same extent. However, it does distinguish between the main groups present in milk and for an investigation such as this it is considered adequate. Micro-organisms not covered by the above scheme were placed in a group termed 'unclassified'. The unclassified group also included isolates which failed to develop, either in the litmus milk or on the surface of agar plates. There are several reasons why some isolates would fail to grow: (1) As a result of technique, i.e. very small sub-surface colonies on the agar plates may become somewhat obscured when the surface of the agar is deformed by the platinum loop at the time of transfer and it is possible that in some cases the colony is not actually transferred. (2) As a result of slow growth, i.e. when transferred from the agar plate to litmus milk the colony may be completely embedded in a small block of agar and during the incubation of the litmus milk the colony may fail to grow out of the agar into the milk. (3) As a result of lack of necessary growth substances in the litmus milk. (4) As a result of unfavourable oxygen tension on the agar surface after streaking from litmus milk.

In an attempt to eliminate no growth due to factor (2) isolates showing no growth in litmus milk after 3 days, as evidenced by lack of growth following a surface agar streak, were incubated at 32°C for another 7 days. After this time they were again streaked on agar plates. This re-incubation of the litmus milk samples did in fact produce subsequent growth on agar plates with several isolates which would otherwise have been placed in the group 'unclassified'.

Results and Discussion

The distribution of the milk samples in relation to the range of colony count is shown in Fig. 9. The choice of the ranges was based on

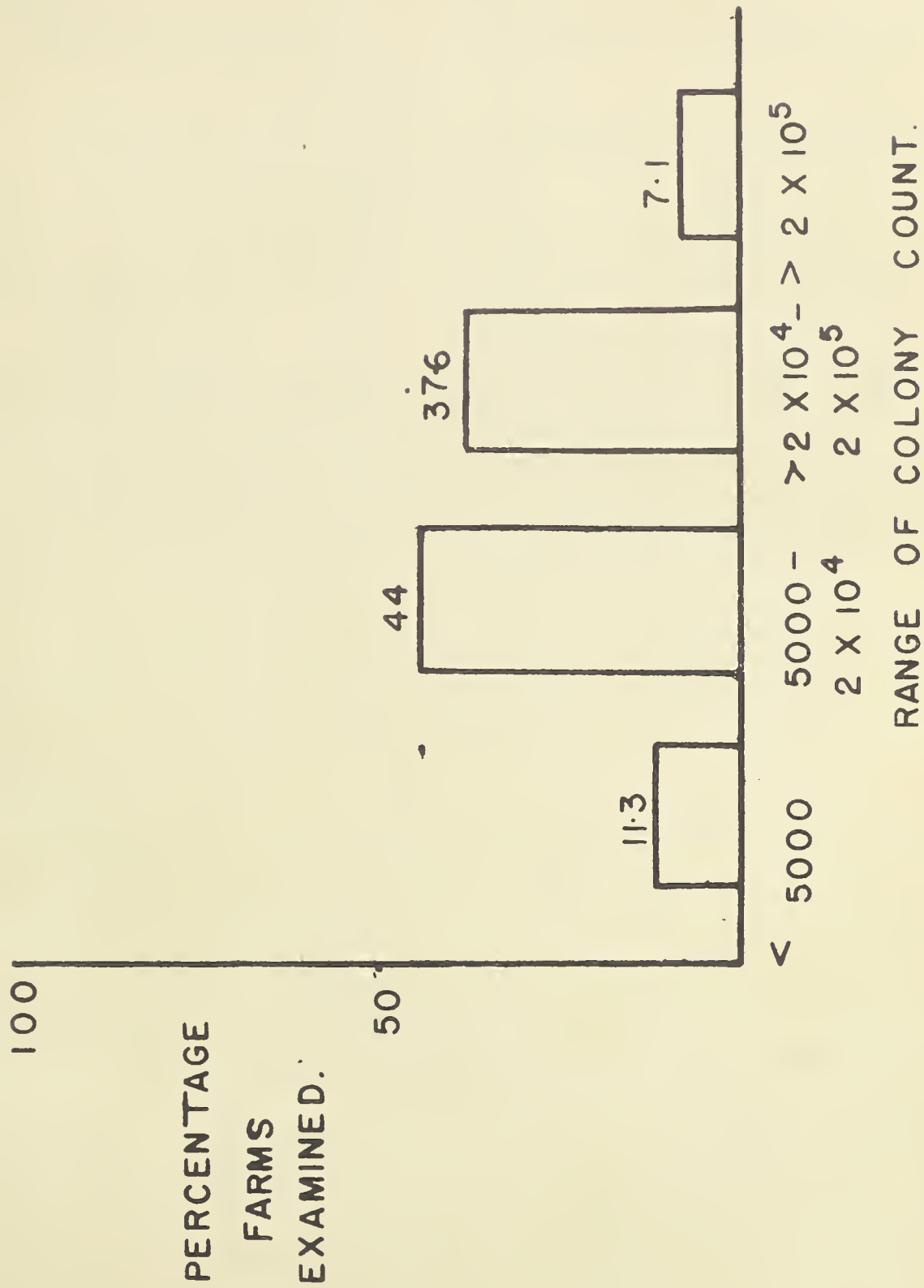


FIGURE 9. Distribution of milk samples in relation to the range of colony count

the observations by Thomas et al. (1962) shown in Table 7.

TABLE 7

Recommended range of colony count in
relation to conditions of milk production

Colony Count Range/ml	Conditions of Production
$< 5 \times 10^3$	Milking equipment is efficiently cleaned
$5 \times 10^3 - 2 \times 10^4$	Satisfactory
$> 2 \times 10^4 - 2 \times 10^5$	Cleaning methods could be improved
$> 2 \times 10^5$	Unsatisfactory

The distribution of the different types of bacteria found in the present work and their relation to the range of colony count is shown in Table 8.

The flora of the milk samples with a colony count < 5000 /ml was found to be dominantly micrococci (67.8%) with streptococci forming the next largest group.

The flora of the milk samples with a colony count between 5000 and 2×10^4 /ml were quite similar to those of the previous group with a slight fall in the percentage of micrococci (54.2%) and a slight increase in the percentage of streptococci (22.8%) and Gram-negative rods (7.9%).

In the next group of samples, with colony counts $> 2 \times 10^4 - 2 \times 10^5$ /ml, the micrococci were no longer the dominant group of micro-organisms, accounting for only 33.8% of the total flora, while the streptococci now accounted for 37.0%. At the same time the Gram-negative rods had become more apparent.

TABLE 8

The incidence of different types of bacteria
according to range of colony count of milk sample

Type of Organism	Percentages and numbers* of cultures isolated from samples with colony counts/ml within the ranges				Totals
	<5000	5000 - 2×10^4	$>2 \times 10^4$ - 2×10^5	$>2 \times 10^5$	
Micrococci	67.8 (217)	54.2 (671)	33.8 (358)	28.0 (560)	46.2 (1302)
Streptococci	16.3 (52)	22.5 (279)	37.0 (392)	13.0 (26)	26.5 (749)
Asporogenous Gram +ve Rods	5.9 (19)	6.3 (78)	7.0 (74)	11.5 (23)	6.9 (194)
Gram -ve Rods	4.1 (13)	7.9 (98)	14.2 (151)	35.5 (71)	11.8 (333)
Bacilli	3.1 (10)	3.5 (43)	2.7 (29)	8.5 (17)	3.5 (99)
Coliforms	0.3 (1)	0.4 (5)	0.6 (6)	0.5 (1)	0.5 (13)
Unclassified	2.5 (8)	5.2 (66)	4.7 (50)	3.0 (6)	4.6 (130)
	320	1240	1060	300	2420

* numbers in brackets

With the colony count range $>2 \times 10^5$ the micrococci (28%) continued to decline, whereas the Gram-negative rods (35.5%) were now the largest group of organisms. This increase in the Gram-negative rods appeared to be mainly at the expense of the streptococci (13.0%).

It appears generally that the percentage of micrococci decreases and the percentage of Gram-negative rods increases with increasing colony count. With regard to the streptococci the picture is not quite so clear,

with an increase in percentage occurring up to a certain range of colony count ($>2 \times 10^4$ - 2×10^5) after which the percentage appears to decline.

Bar graphs (Fig. 10) of the data from Table 8 and the corresponding data from Thomas et al. (1962) have been constructed for ease of comparison. The data are somewhat modified as Thomas et al. subdivided the Gram-negative rods and did not subdivide the Gram-positive non-spore-forming rods, whereas in the investigation reported in this paper the situation was the reverse. Generally there is a marked similarity between the two sets of data.

The general trends described above and illustrated in Fig. 10 would appear to apply only to the pooled results of many milk samples. A study of the flora of the individual milk samples reveals very marked variation even within any one particular range of plate count. This is illustrated in Fig. 11 which shows the percentage of micrococci plotted against the log of the colony count. The correlation coefficient of $r = -0.59$ indicates a fair degree of correlation, however because of the wide variation found with individual samples it would not be possible to forecast one value from the other.

A further comparison of the data of Thomas et al. (1962) and those found in the present investigation is made in Table 9. This table shows the incidence of milk samples in which the different types of bacteria were detected and in which these types constituted 50% or more of the flora.

The results of the two investigations under discussion are very similar, the major difference being accounted for by the inclusion of more samples in the high colony count range by Thomas et al. (1962). There seems to be no explanation, however, for the differences found in the percentages of streptococci, the values given by Thomas et al. being consistently lower than the values found in this work except for the class $>2 \times 10^5$.

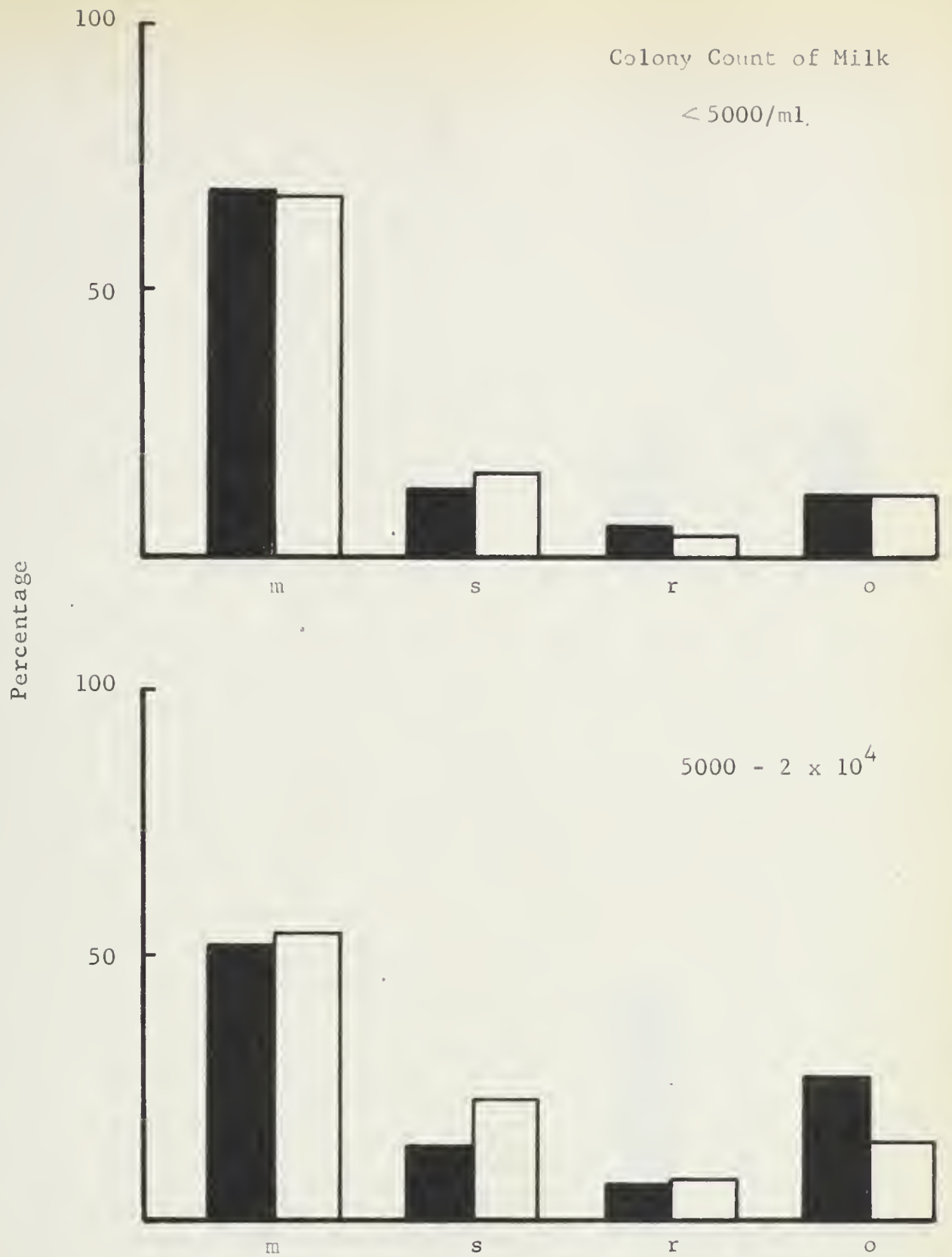


FIGURE 10. The flora of raw milk in relation to range of colony count



After Thomas et al. (1962)



Edmonton

m - micrococci
s - streptococci
r - Gram - ve rods
o - others

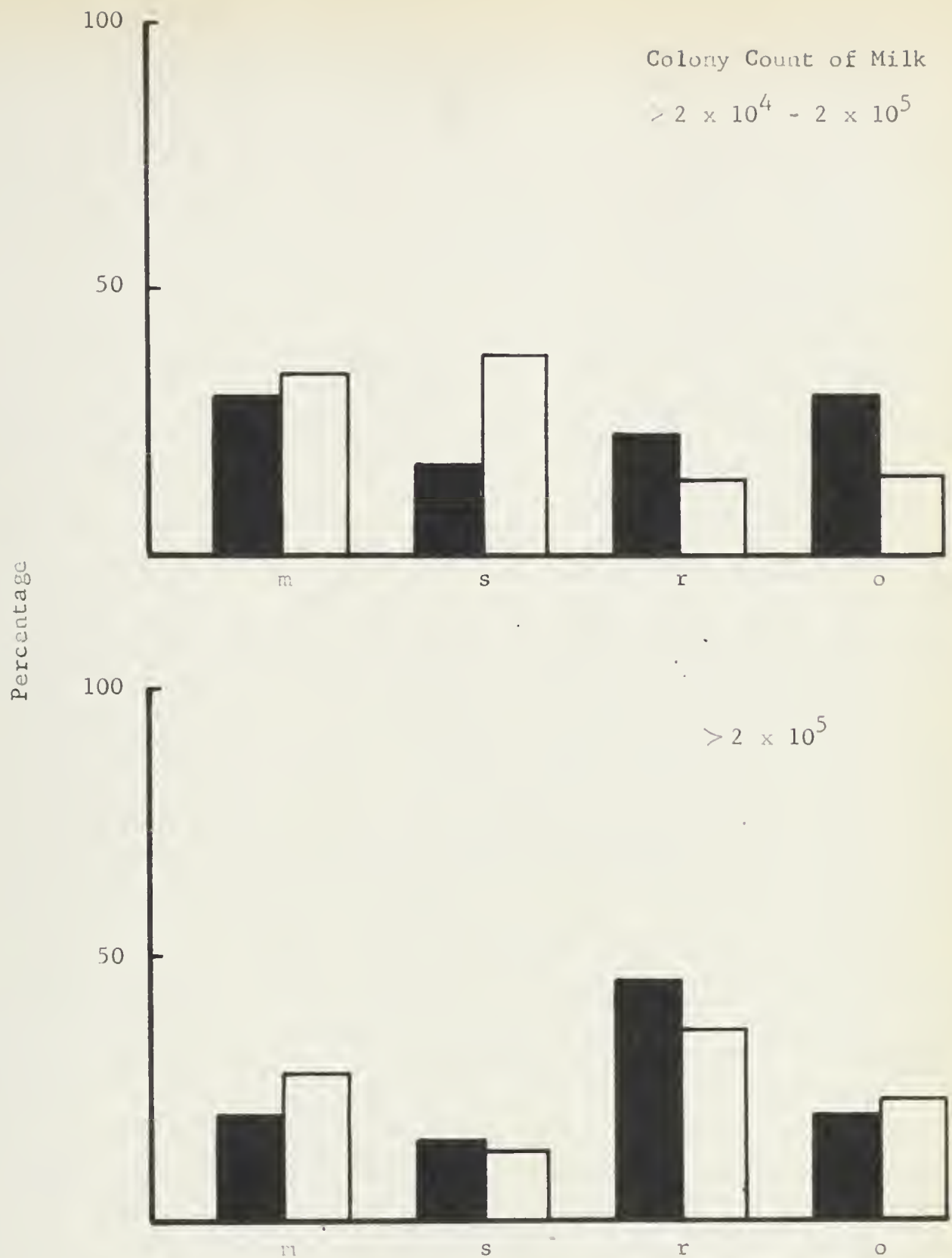


FIGURE 10. (cont.) The flora of raw milk in relation to range or colony count

■ After Thomas *et al.* (1962)

□ Edmonton

m - micrococci
s - streptococci
r - Gram - ve rods
o - others

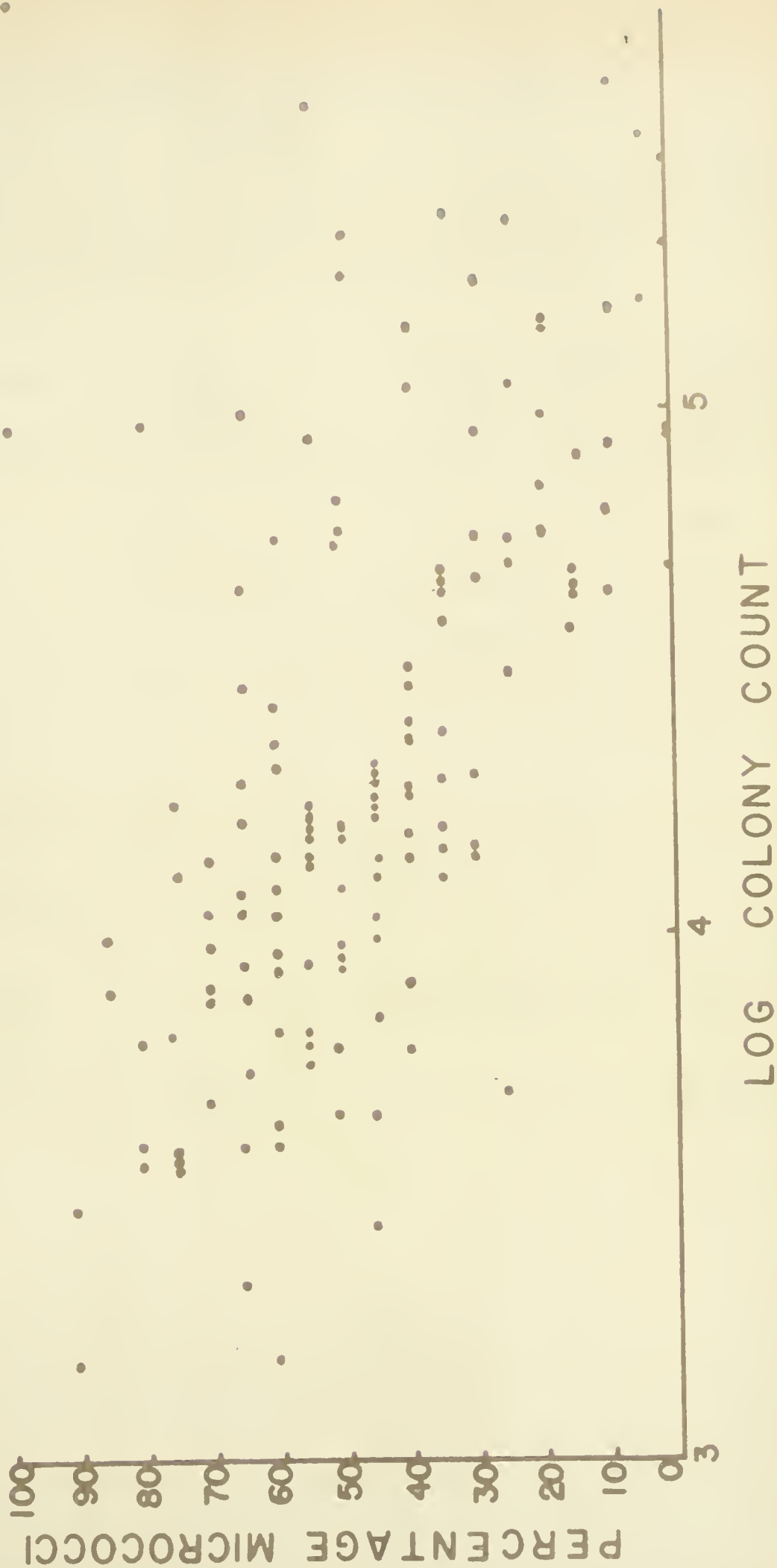


FIGURE 11. The relationship between the percentage micrococci and the log colony count of milk

The samples studied by Thomas et al. were incubated at 30°C, whereas in the present investigation the samples were incubated at 32°C. However, this small difference is probably not important.

TABLE 9

The incidence of milk samples in which the various bacterial types were detected (a), and in which they constituted 50% or more of the flora (b)

After Thomas <u>et al.</u> (1962)			Results at Edmonton (1962)		
Bacterial Types	Percentages of Samples		Bacterial Types	Percentages of Samples	
	(a)	(b)		(a)	(b)
Micrococci	90.8	43.7	Micrococci	95.7	49.6
Streptococci	77.0	11.5	Streptococci	87.2	16.3
Asporogenous Gram + ve rods	64.3	4.6	Asporogenous Gram + ve rods	56.7	1.4
Coli-Aerogenes Bacteria	19.5	6.9	Coliforms	8.5	
Pigmented Gram -ve rods	51.7	6.9	Gram -ve rods	58.1	5.7
Non-Pigmented Gram -ve rods	52.9	11.5			
Aerobic Spore Forming rods	29.8	4.6	Sporeforming rods	34.7	0.7
Streptomyces	5.7				

Section 3

The Flora of Raw Milk Before and After Preliminary Incubation, with some Observations on the Resazurin Test

Methods

During the months of March to June, 1963, milk samples were collected from 39 farms by milk truck drivers (Silverwood's Dairies Limited) and placed in refrigerated containers. The milk at the time of collection was 48 hr old (i.e. two days' milkings.). The samples were collected from the dairy and brought in an ice box to the laboratory on the same day. The milk was then divided into 4 sub-samples, each of 10 ml. Resazurin solution (1 ml) was added to two of the tubes. One tube of milk and another tube containing milk plus resazurin were placed in a water bath at 55°F for 18 hr. The two remaining tubes were kept in the refrigerator overnight.

The following day the tubes containing resazurin were incubated at 35°C and observed every hour for 9 hr. The Munsell P7/4 colour standard was used as an end-point. The reduction time of samples which had not reached this end-point after 9 hr was arbitrarily made 10 hr.

The samples of fresh milk and P.I. milk were plated on standard plate count agar. Following incubation at 32°C for 48 hr the colonies were counted and colonies were picked from the plates in the same way as described in Section 1. One modification was made, 25 colonies were selected for picking (by means of a 5 x 5 line grid) instead of 20 from each plate as was done in the earlier work. The scheme shown in Figure 8 was used for identification of the isolates.

Results and Discussion

The results of the analysis of the flora before and after P.I. are represented in the form of bar graphs (Fig. 12) for ease of interpretation. The precise data for each sample is recorded in Appendix A. The

Sample

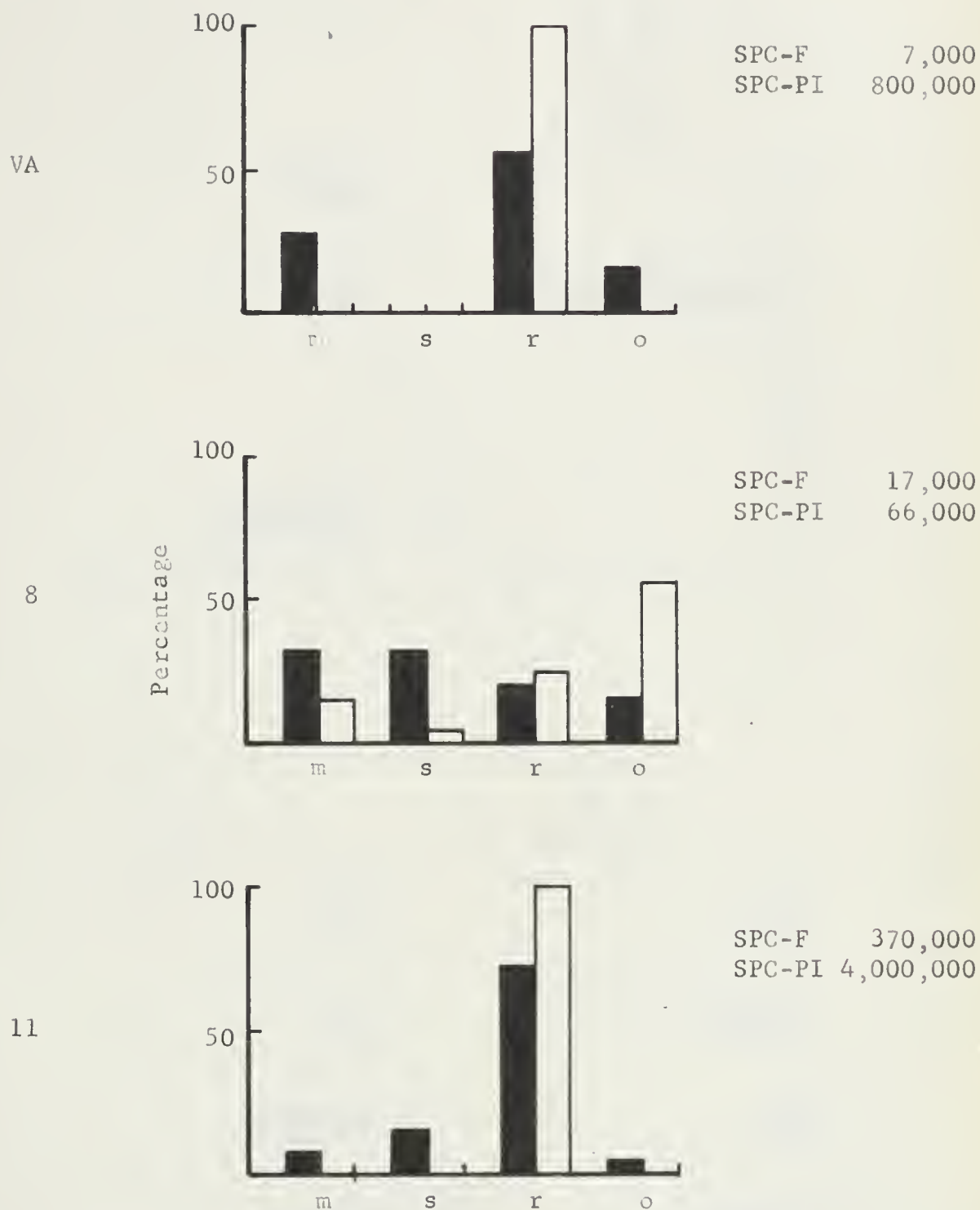


FIGURE 12. The relationship between the flora of milk before and after preliminary incubation (P.I.) at 55°F (12.8°C) for 18 hr

■ Before P.I.
 □ After P.I.

m - micrococci
 s - streptococci
 r - Gram - ve rods
 o - others

SPC-F - Standard plate count fresh
 SPC-PI - Standard plate count after P.I.



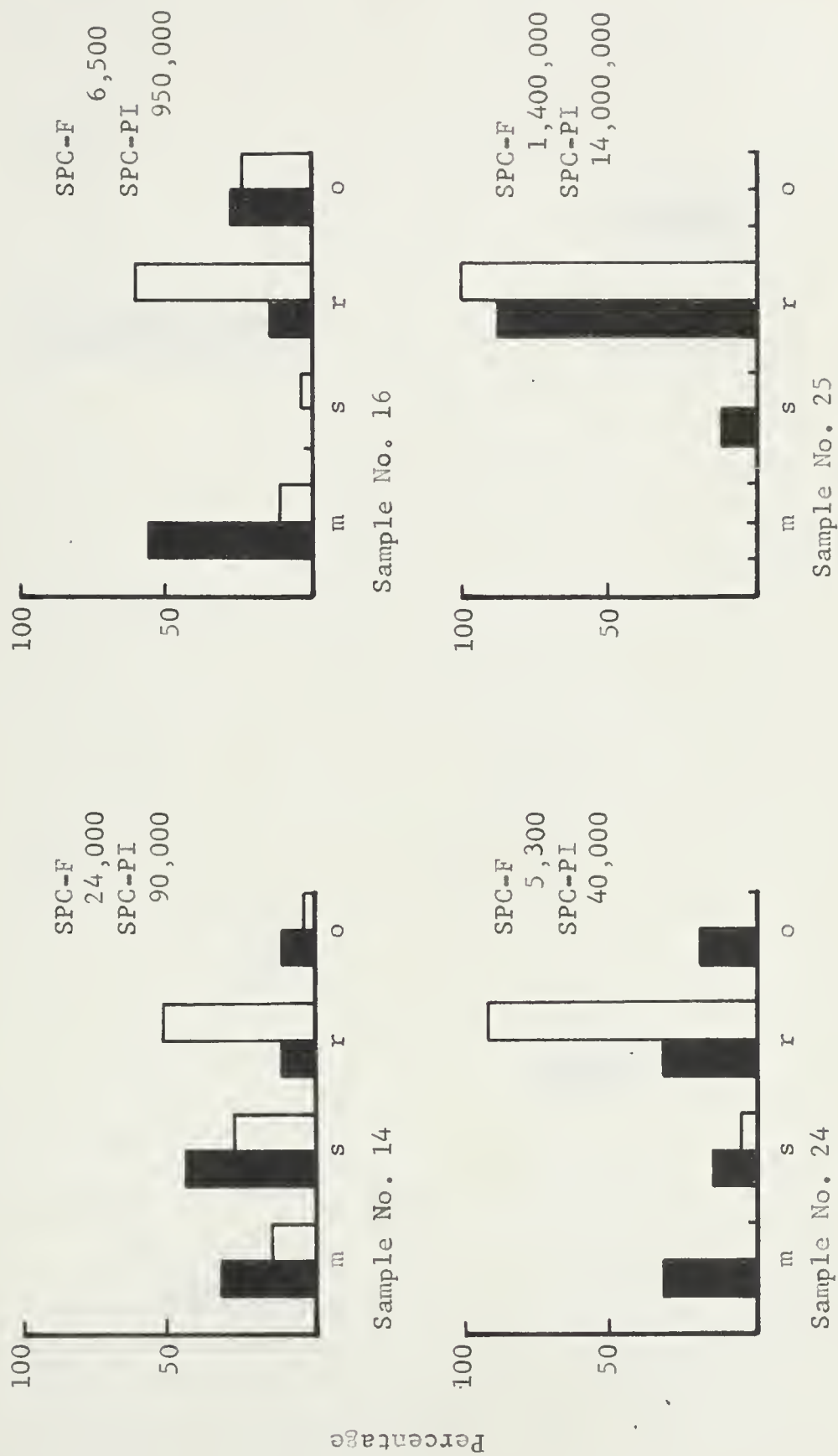


FIGURE 12. (cont.) The relationship between the flora of milk before and after preliminary incubation (P.I.) at 55°F (12.8°C) for 18 hr

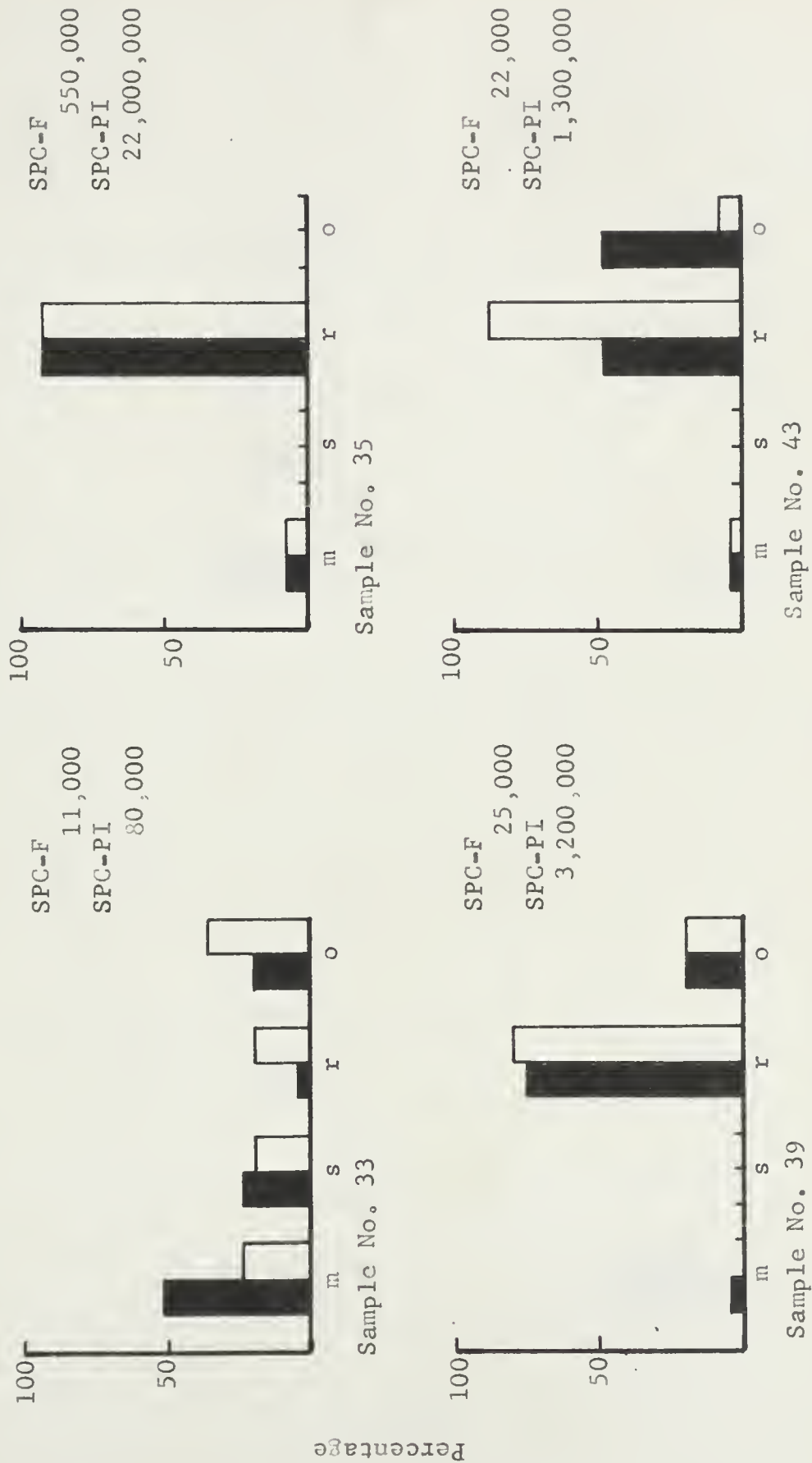


FIGURE 12. (cont.) The relationship between the flora of milk before and after preliminary incubation (P.I.) at 55°F (12.8°C) for 18 hr

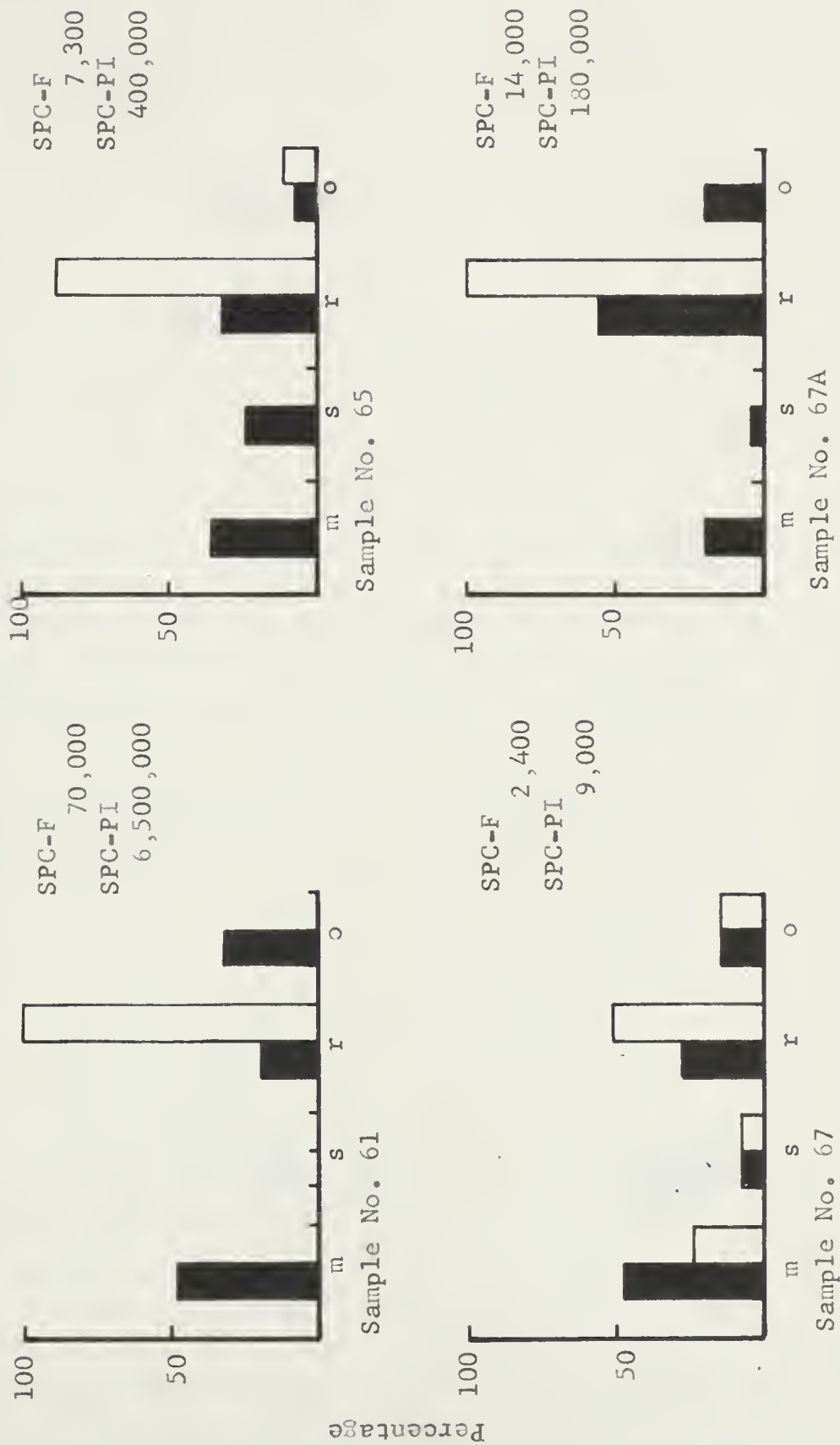


FIGURE 12. (cont.) The relationship between the flora of milk before and after preliminary incubation (P.I.) at 55°F (12.8°C) for 18 hr

Before P.I. SPC-F - Standard plate count fresh
After P.I. SPC-PI - Standard plate count after P.I.

m - micrococci
s - streptococci
r - Gram - ve rods
o - others

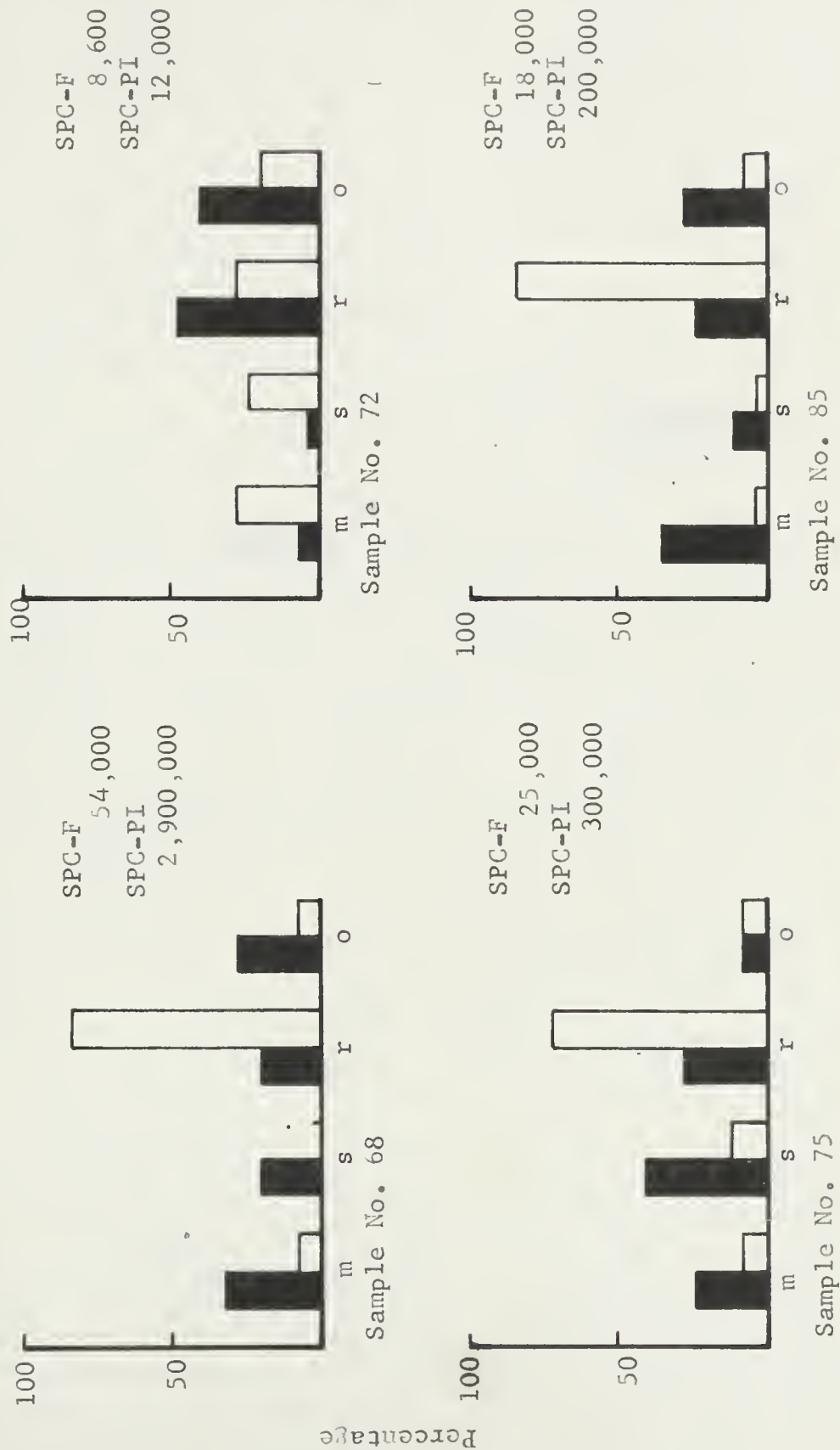


FIGURE 12. (cont.) The relationship between the flora of milk before and after preliminary incubation (P.I.) at 55°F (12.8°C) for 18 hr

Before P.I. m - micrococci SPC-F - Standard plate count fresh
After P.I. s - streptococci SPC-PI - Standard plate count after P.I.
r - Gram - ve rods
o - others

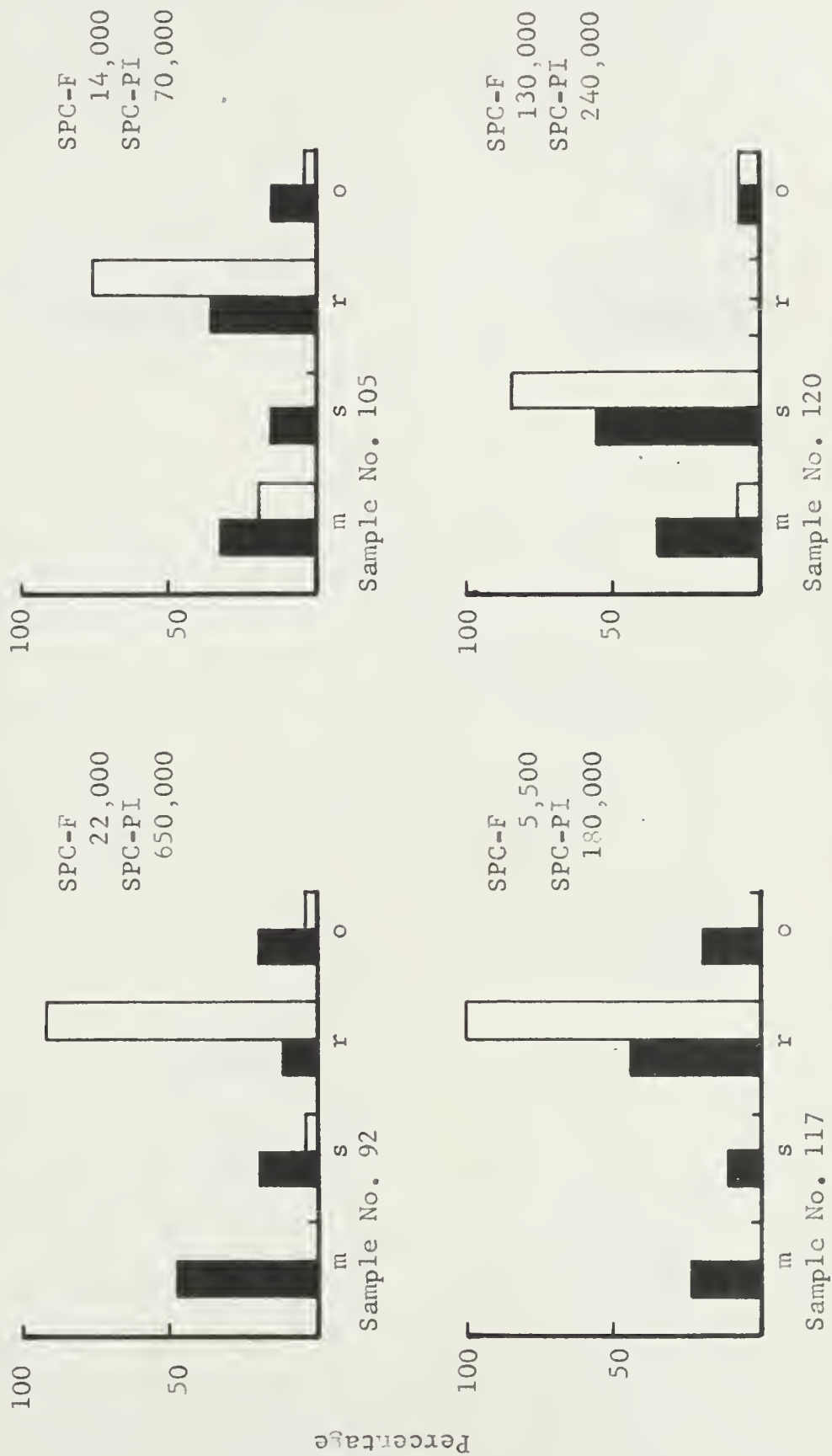


FIGURE 12. (cont.) The relationship between the flora of milk before and after preliminary incubation (P.I.) at 55°F (12.8°C) for 18 hr

Before P.I. After P.I.

m - micrococci SPC-F - Standard plate count fresh

s - streptococci SPC-PI - Standard plate count after P.I.

r - Gram - ve rods

o - others

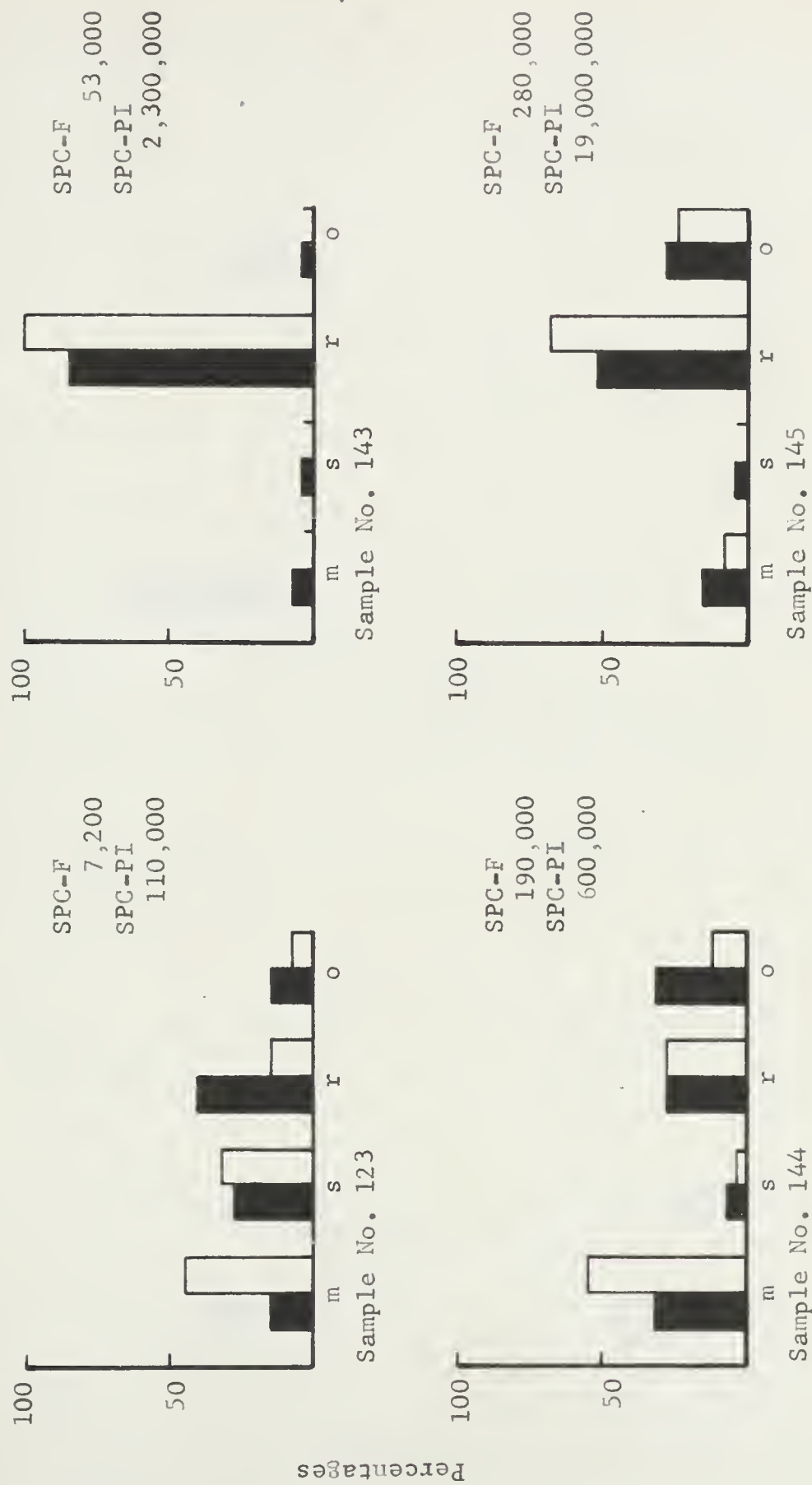


FIGURE 12. (cont.) The relationship between the flora of milk before and after preliminary incubation (P.I.) at 55°F (12.8°C) for 18 hr

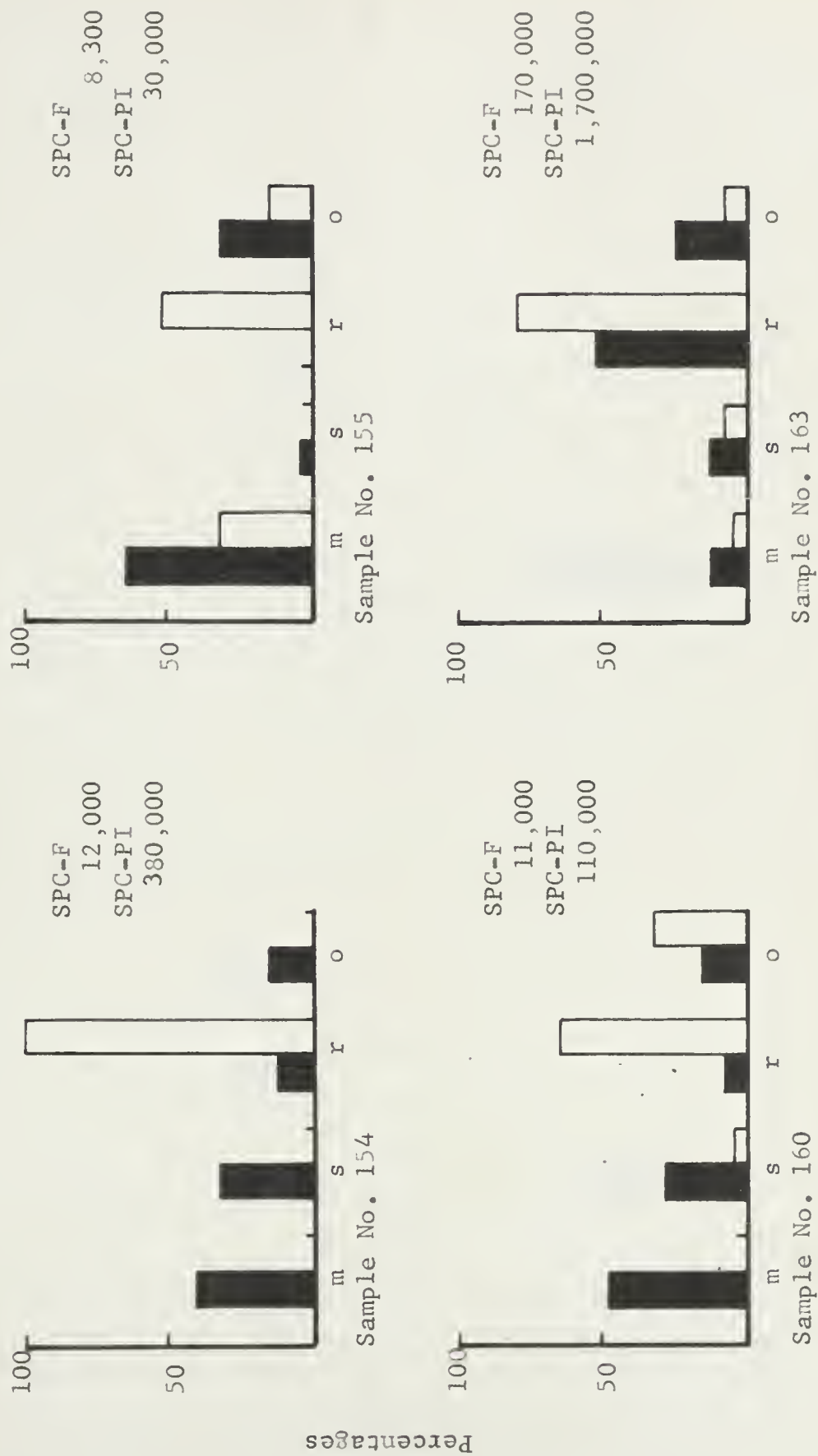


FIGURE 12. (cont.) The relationship between the flora of milk before and after preliminary incubation (P.I.) at 55°F (12.8°C) for 18 hr

Before P.I. SPC-F - Standard plate count fresh
After P.I. SPC-PI - Standard plate count after P.I.

m - micrococci
s - streptococci
r - Gram - ve rods
o - others



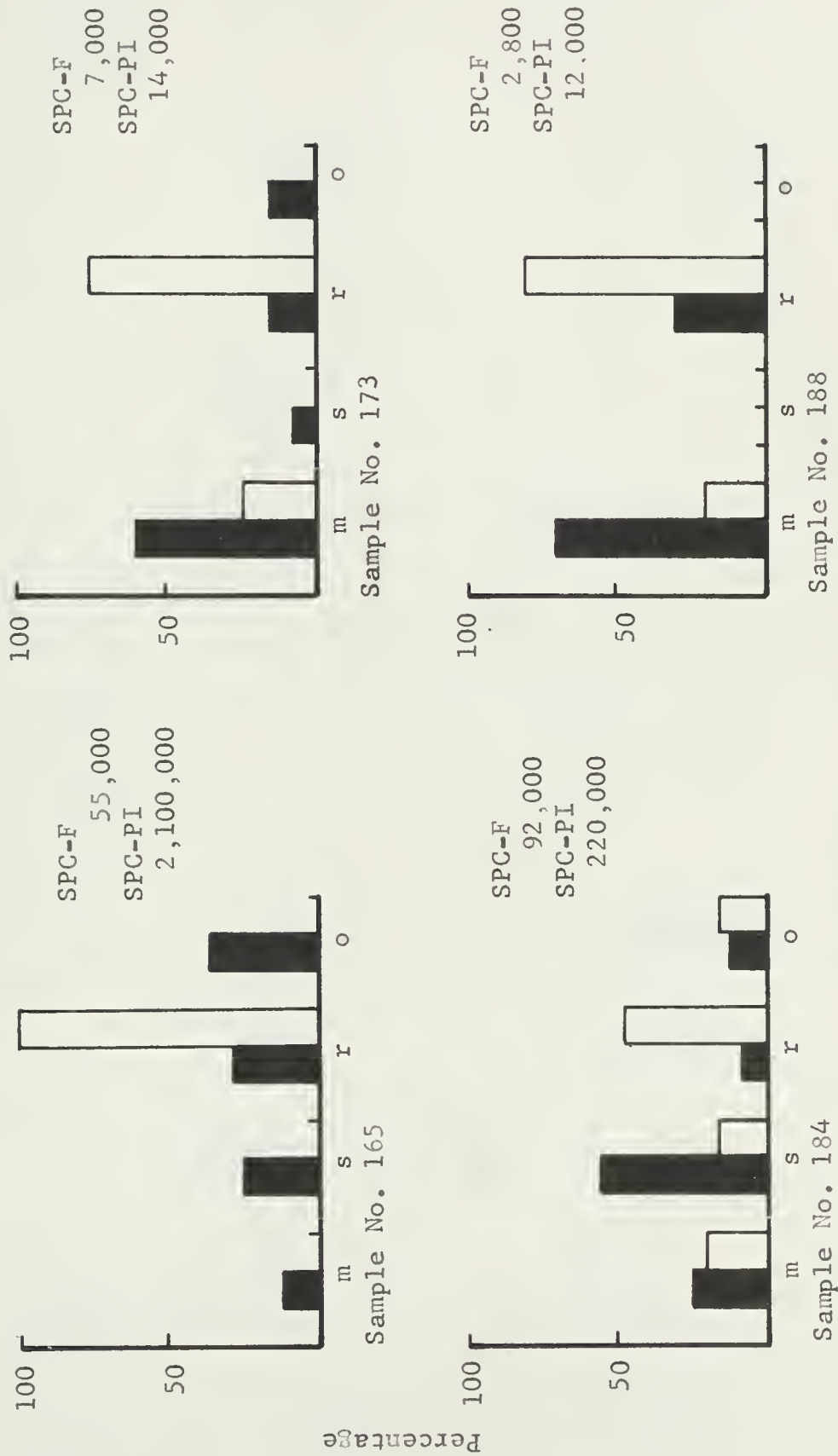


FIGURE 12. (cont.) The relationship between the flora of milk before and after preliminary incubation (P.I.) at 55°F (12.8°C) for 18 hr

Before P.I. m - micrococci
After P.I. s - streptococci
 r - Gram - ve rods
 o - others

SPC-F - Standard plate count fresh
SPC-PI - Standard plate count after P.I.

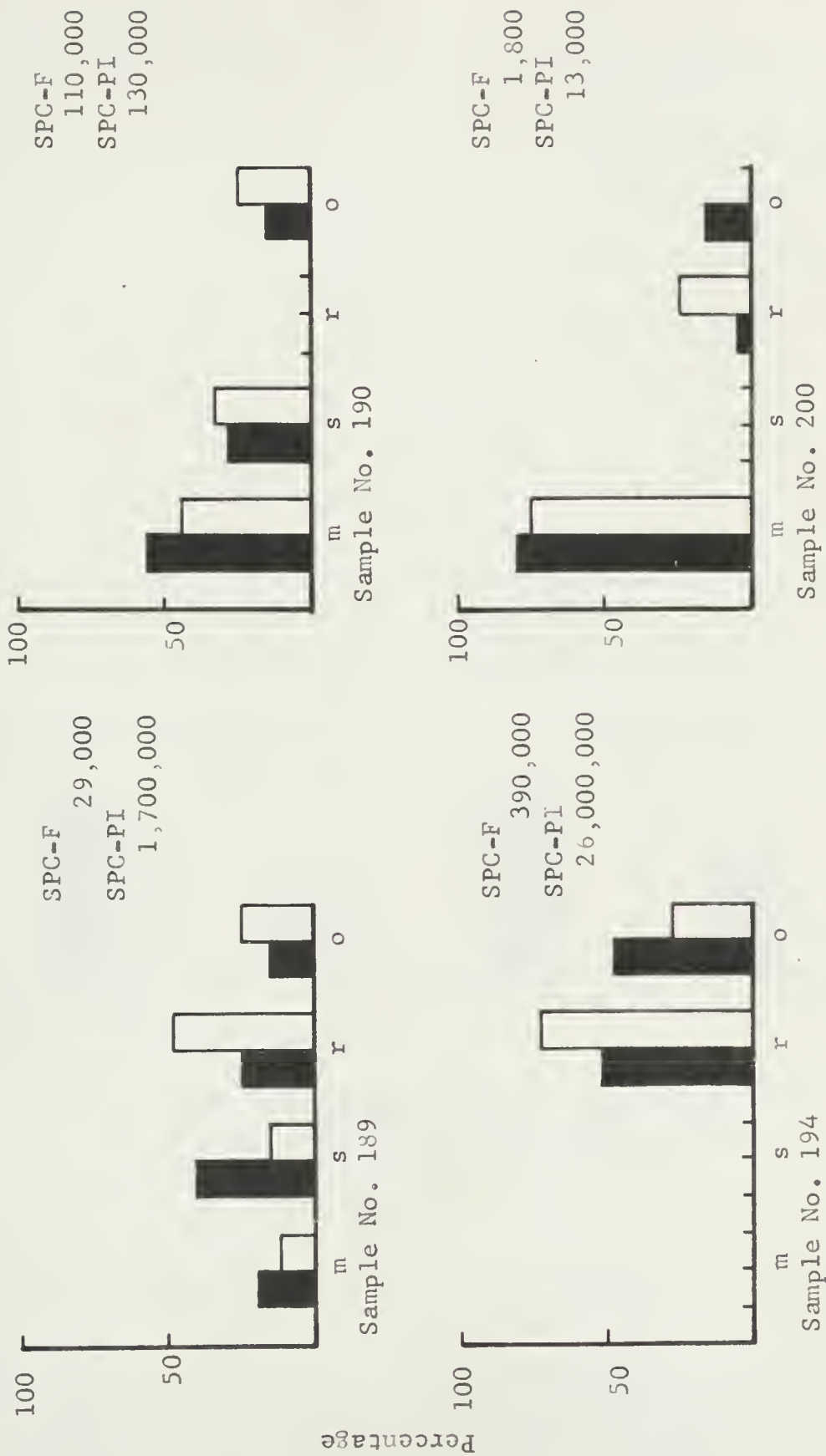


FIGURE 12. (cont) The relationship between the flora of milk before and after preliminary incubation (P.I.) at 55°F (12.8°C) for 18 hr

Before P.I. After P.I.

m - micrococci
s - streptococci
r - Gram - ve rods
o - others

SPC-F - Standard plate count fresh
SPC-PI - Standard plate count after P.I.

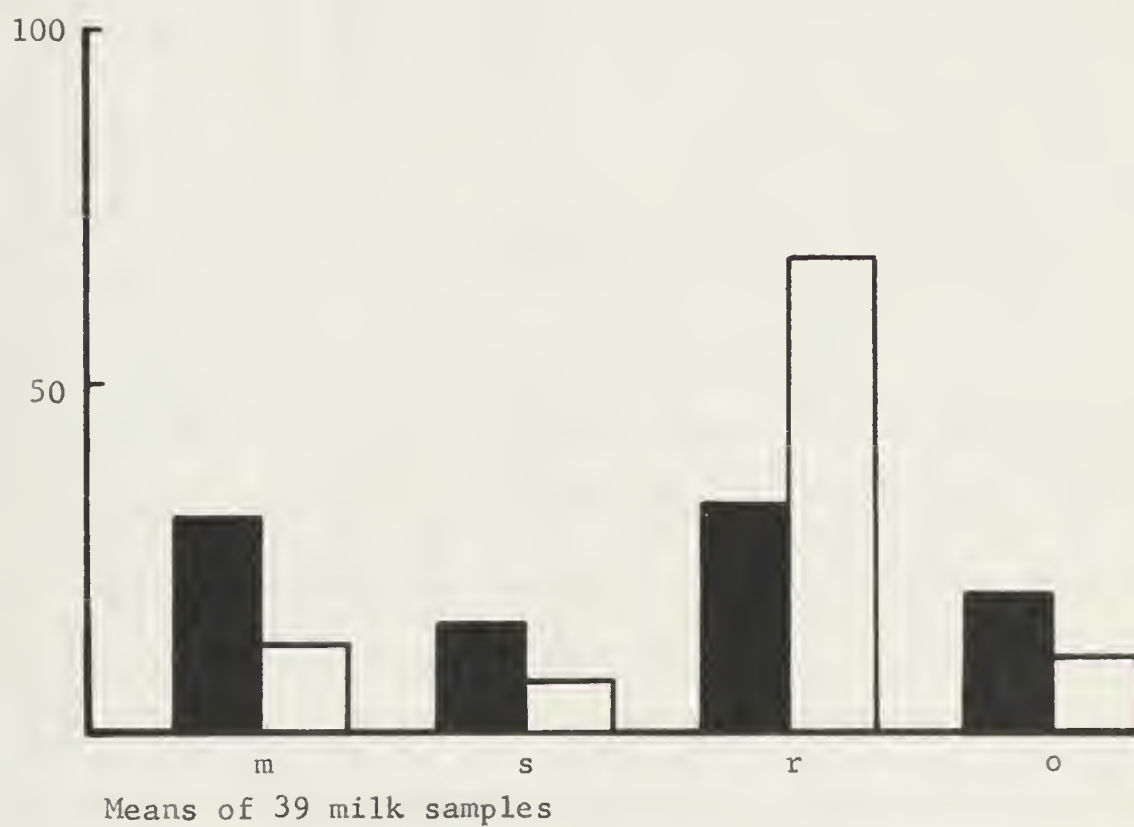


FIGURE 12. (cont.) The relationship between the flora of milk before and after preliminary incubation (P.I.) at 55°F (12.8°C) for 18 hr

■ Before P.I.
□ After P.I.

m - micrococci
s - streptococci
r - Gram - ve rods
o - others

first 39 bar graphs represent individual milk samples. The last one represents the average.

From the bar graphs, Figure 12, it can be seen that the percentage of micrococci before P.I. is usually higher than after P.I. It can also be seen that the percentage of Gram-negative rods is higher after P.I. than before. This would be expected from a consideration of the data on growth temperatures in Bergey's Manual (1957). For the family Micrococcaceae the minimum growth temperatures would appear to be close to 10°C with the exception of one species, namely Micrococcus cryophilus. The work of McLean, Sulzbacher and Mudd (1951) showed that this remarkable organism had an optimum growth temperature of 9.8°C with a range from -4.0°C to 23 or 24°C . It is not very difficult to understand the increase in the percentage of Gram-negative rods following P.I. as many of these have minimum growth temperatures below 10°C . As ability to grow at 10°C and/or 45°C is one of the main criteria for the classification of streptococci, one would expect the effect of P.I. on the change in the percentage of streptococci in milk to be unpredictable. This can be seen from the bar graphs. Thus, if the streptococci in the milk were mainly of the pyogenic group and the viridans group, both of which do not grow at 10°C , one would expect little multiplication on P.I. However, if the streptococci were of the enterococcus group or the lactic group, which grow at 10°C , multiplication might take place during P.I.

The results of the analysis of the flora before and after P.I. appear to be in agreement with data on the growth temperatures of micrococci, streptococci, and Gram-negative rods. In view of this it would be expected that the increase in numbers of micro-organisms following P.I. would be related to the percentages of the various groups present in the

fresh milk. The results of the standard plate count (S.P.C.) before and after P.I. (see Fig. 12) indicate a limited relationship. It is suggested that the increase in S.P.C. following P.I. will be more closely related to the percentage of micro-organisms capable of growth at the temperature of P.I. and not to any one particular group of micro-organisms. The nutritional value of milk as a medium for growth of various micro-organisms was studied by Gyllenberg et al. (1959). These workers found that although some Gram-negative rods (Flavobacterium spp. and Alcaligenes bookeri) were essentially psychophilic, as compared with Micrococcus spp., the latter grow more quickly in milk even at 5°C. It was concluded that milk is not a good substrate for the former, but is for the latter. Gibson and Abd-el Malek (1957) had stated earlier that the nutritional value of the milk itself probably plays a part in determining which organisms are able to grow. The results of these workers and the results of the present investigation would suggest that the increase in the standard plate count of milk following P.I. is related to the growth temperatures and the nutritional requirements of the micro-organisms present rather than to the presence of one particular group of micro-organisms (micrococci, streptococci, Gram-negative rods etc.). Inhibitory substances in the milk may play a part.

The resazurin tests and leucocyte counts were made on these milk samples in the hope that this would give some information on the effect of the different types of bacteria on the reduction of resazurin. In an attempt to unravel the effects of numbers and percentages of bacteria on the resazurin test a graph was plotted of resazurin reduction time versus the logarithm of the standard plate count, the individual points on the graph being marked differently according to the type of bacteria forming the largest percentage in the milk sample. This is illustrated in Figure 13. The data used are for milk before and after P.I.

The graph shows that for all samples in which streptococci were the dominant group of organisms the points fall below the regression line. This may be interpreted as meaning that the streptococci are very active in the dye reduction tests. The one sample in which coliforms were dominant falls below the line, again indicating very active reduction of resazurin. For Gram-negative rods more than half of the points are above the regression line indicating that many Gram-negative rods are not active in the reduction of resazurin. With the micrococci the points are evenly distributed on both sides of the line indicating a fair degree of activity in the reduction of resazurin. However, not all micrococci and streptococci should be regarded as active reducers of resazurin and not all Gram-negative rods should be regarded as inactive reducers of resazurin. The regression line has been drawn merely for the purpose of discussion and its inclusion does not imply that a prediction of one variable from the other is possible. A regression line as an aid to prediction is of little value when the correlation coefficient is less than 0.85. The correlation coefficient of the data in Figure 13 is obviously less than this. The trends noted above are in agreement with the results of Wilson et al. (1935) Hobbs (1939), Thomé (1941) and Garvie and Rowlands (1952b). Wilson et al. (1935) determined the reducing activity of several pure cultures of bacteria at 37°C with methylene blue. On the basis of the results the organisms in decreasing order of activity were: Bact. coli, Bact. aerogenes, streptococci (lactic type), streptococci (unknown type), Staph. aureus, Staph. albus, small micrococci, large micrococci, large Gram-positive rods and Achromobacterium spp. Hobbs (1939) found that the coliforms were the most active dye reducers, followed by Str. lactis and some faecal streptococci, Staph. aureus, Staph. albus, haemolytic streptococci (Group C), some strains

of Str. agalactiae and aerobic spore-bearers. Thomé (1941) reported that the reducing ability of the streptococci, micrococci and coli-aerogenes bacteria was essentially the same. Staphylococci, some micrococci, coli-aerogenes and some streptococci (Group D) were active dye reducers at 37.5°C according to Garvie and Rowlands (1952b). At the same temperature achromobacteria, chromobacteria, Gram-positive rods, microbacteria, and streptococci (Groups B and E) were inactive. At 22°C all the cultures active at 37.5°C were active as were achromobacteria, some micrococci and streptococci (Groups B and E). They suggest that differences in dye reduction tests at 37.5°C and 22°C, on the same milk sample, may be explained in terms of differences in the initial flora. In an earlier publication, Garvie and Rowlands (1952a) stated that the achromobacteria and microbacteria played little part in dye reduction tests. Thus the rate of dye reduction depends not only on the numbers but also on the types of bacteria present in milk. The predominance of the different types depends on conditions of production and subsequent storage of the milk.

Section 4

The Flora of Raw Bulk Tank Milk Before and After P.I. and the Flora of Milking Units

The possibility of using the ratio of SPC after P.I.:SPC before P.I. as an index of the sanitary conditions under which milk has been produced has been discussed earlier. In order to evaluate this possibility further, milk samples were collected from 18 farms during the months of June and July, 1963, and at the same time pulsating rinses were made of the milking units.

Methods

With the following additions the methods were the same as in Section 3. A coagulase test was done on the micrococci, and all Gram-positive cocci were streaked on sheep blood agar plates.

Results and Discussion

The results of the analysis of the flora before and after P.I. given in Figure 14 and in detail in Appendix B again show that the main effect of P.I. is to increase the percentage of Gram-negative rods and reduce the percentage of micrococci in the milk.

The analysis of the flora of the milking equipment is shown below in the form of bar graphs (Fig. 15) and in more detail in Appendix B. The bar graphs combine the data of the flora of the raw milk and the flora of the milking units.

Thomas et al. (1963) isolated and identified bacteria from 158 rinses of milking equipment. Rinses were taken from milking equipment sanitized by various means allowing a comparison of the effects of sanitizing procedures on the flora. Micrococci were dominant when steam, hypochlorite and immersion cleaning were used, and Gram-negative rods were dominant when quaternary ammonium compounds (Q.A.Cs.) were used. When

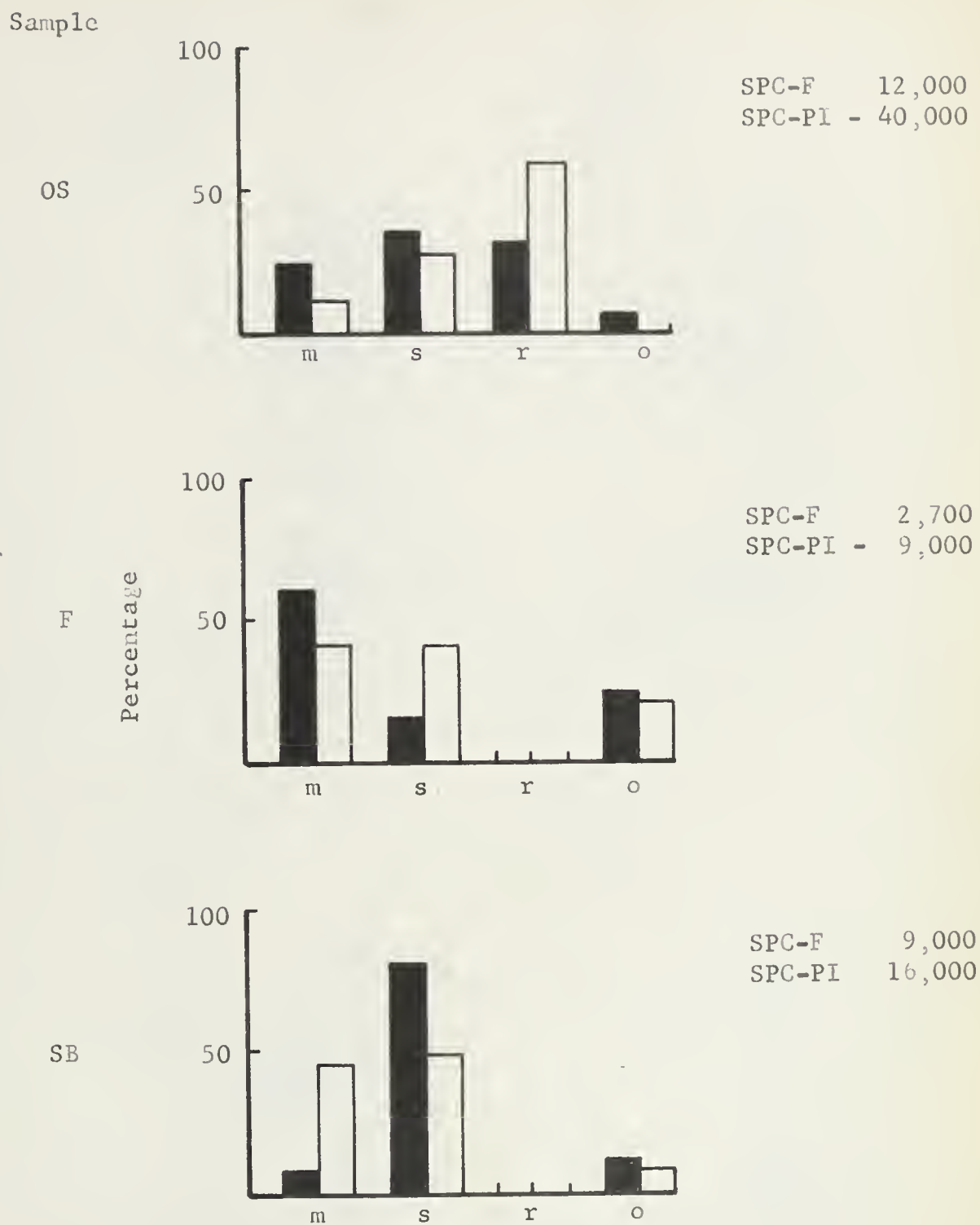


FIGURE 14. The relationship between the flora of milk before and after preliminary incubation (P.I.) at 55°F (12.8°C) for 16 hr

■ Before P.I.	m - micrococci	SPC-F - Standard plate count fresh
□ After P.I.	s - streptococci	SPC-PI - Standard plate count after P.I.
	r - Gram - ve rods	
	o - others	

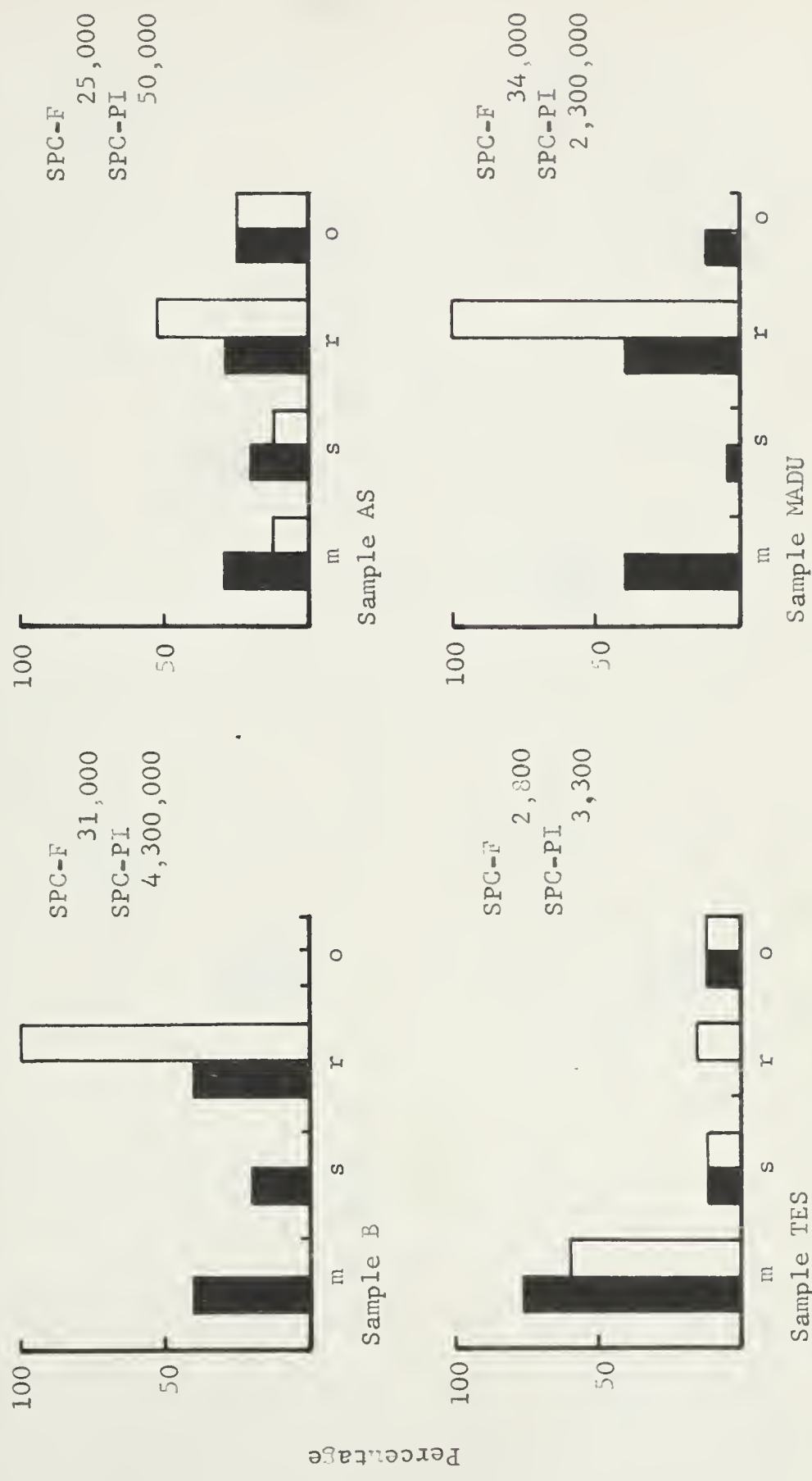


FIGURE 14. (cont.) The relationship between the flora of milk before and after preliminary incubation (P.I.) at 55°F (12.8°C) for 18 hr

Before P.I. SPC-F - Standard plate count fresh
After P.I. SPC-PI - Standard plate count after P.I.

m - micrococci
s - streptococci
r - Gram - ve rods
o - others

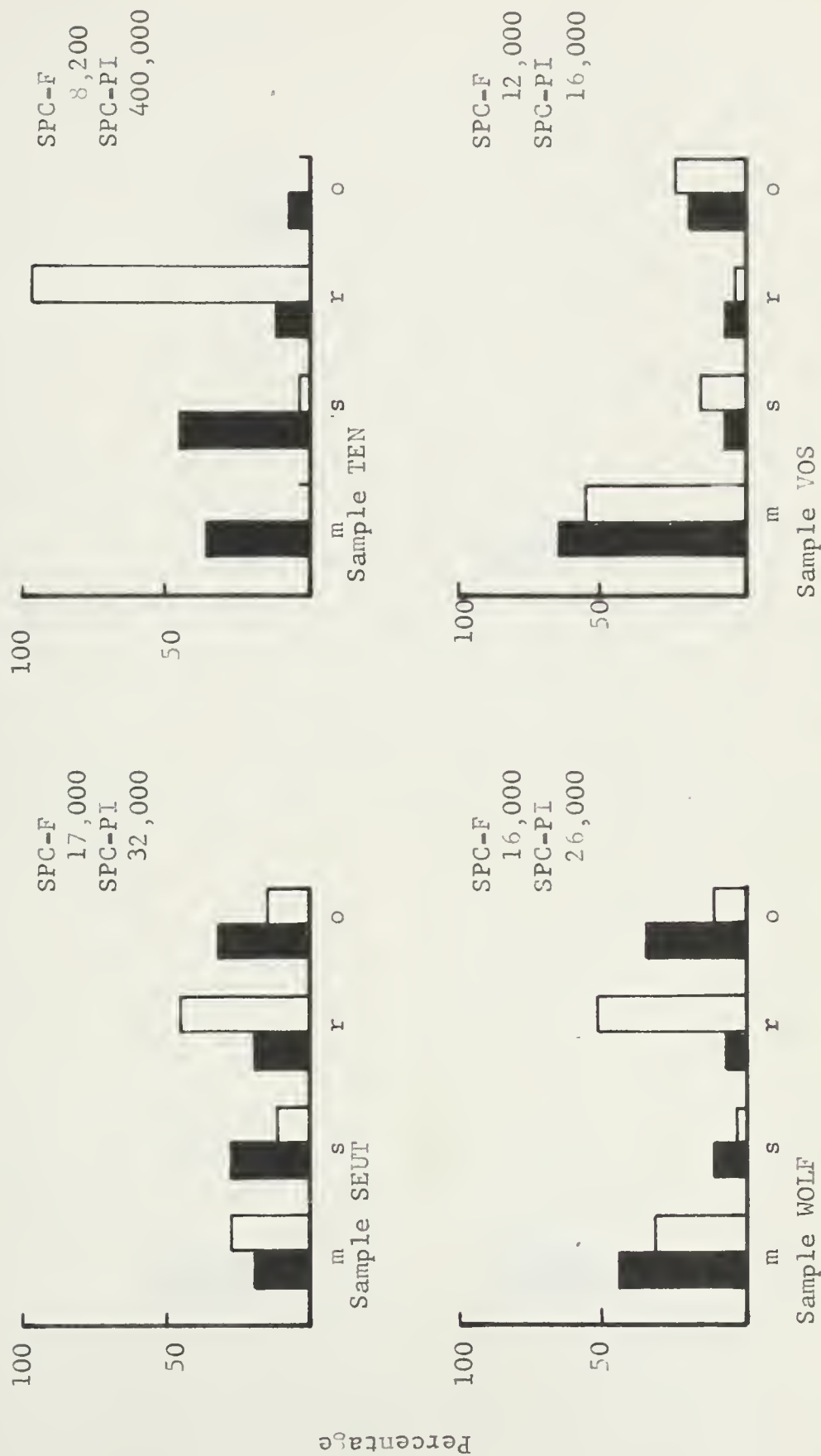


FIGURE 14. (cont.) The relationship between the flora of milk before and after preliminary incubation (P.I.) at 55°F (12.8°C) for 16 hr

Before P.I. m - micrococci
After P.I. s - streptococci
 r - Gram - ve rods
 o - others

SPC-F - Standard plate count fresh
SPC-PI - Standard plate count after P.I.

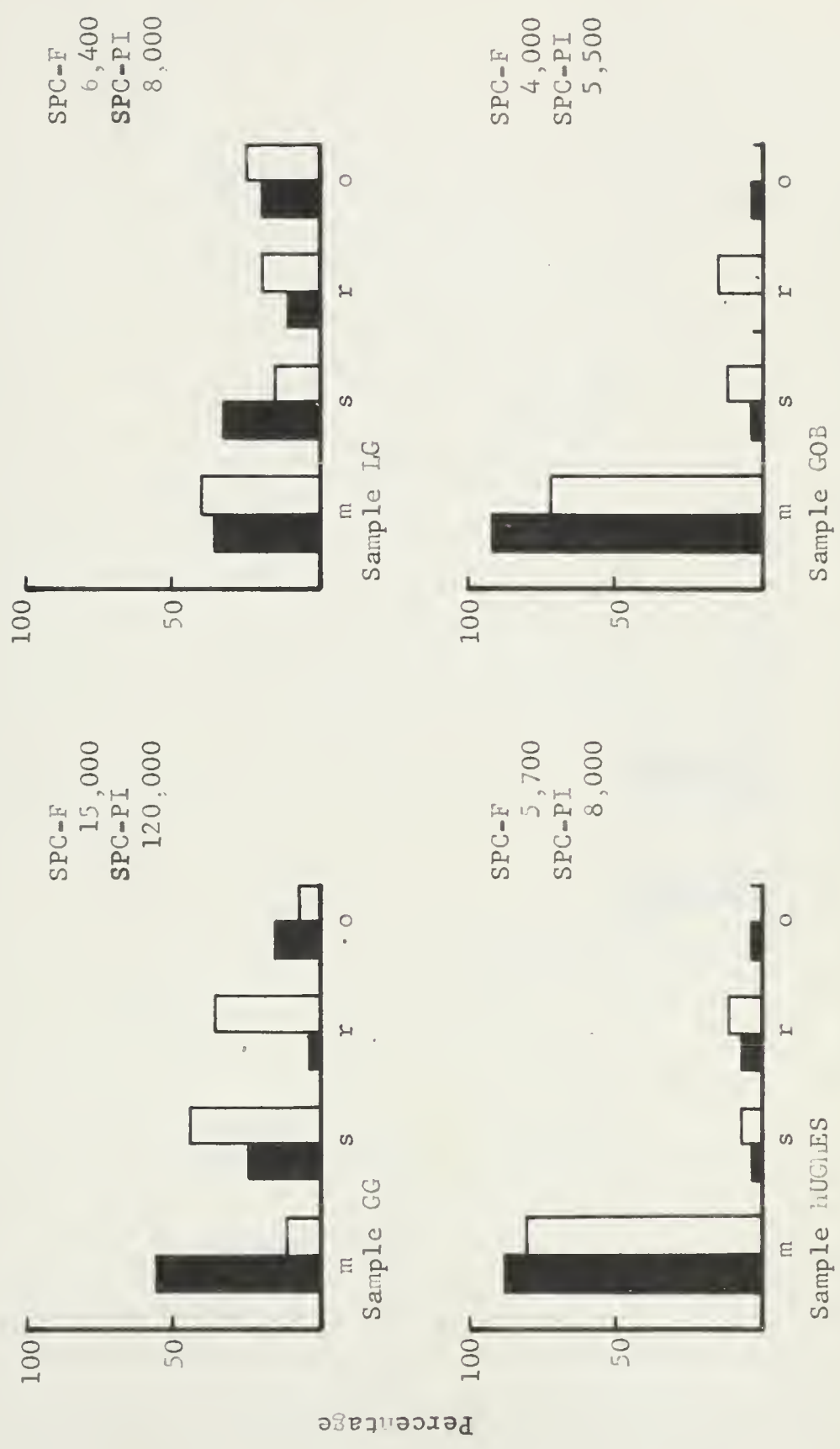


FIGURE 14. (cont.) The relationship between the flora of milk before and after preliminary incubation (P.I.) at 55°F (12.8°C) for 18 hr

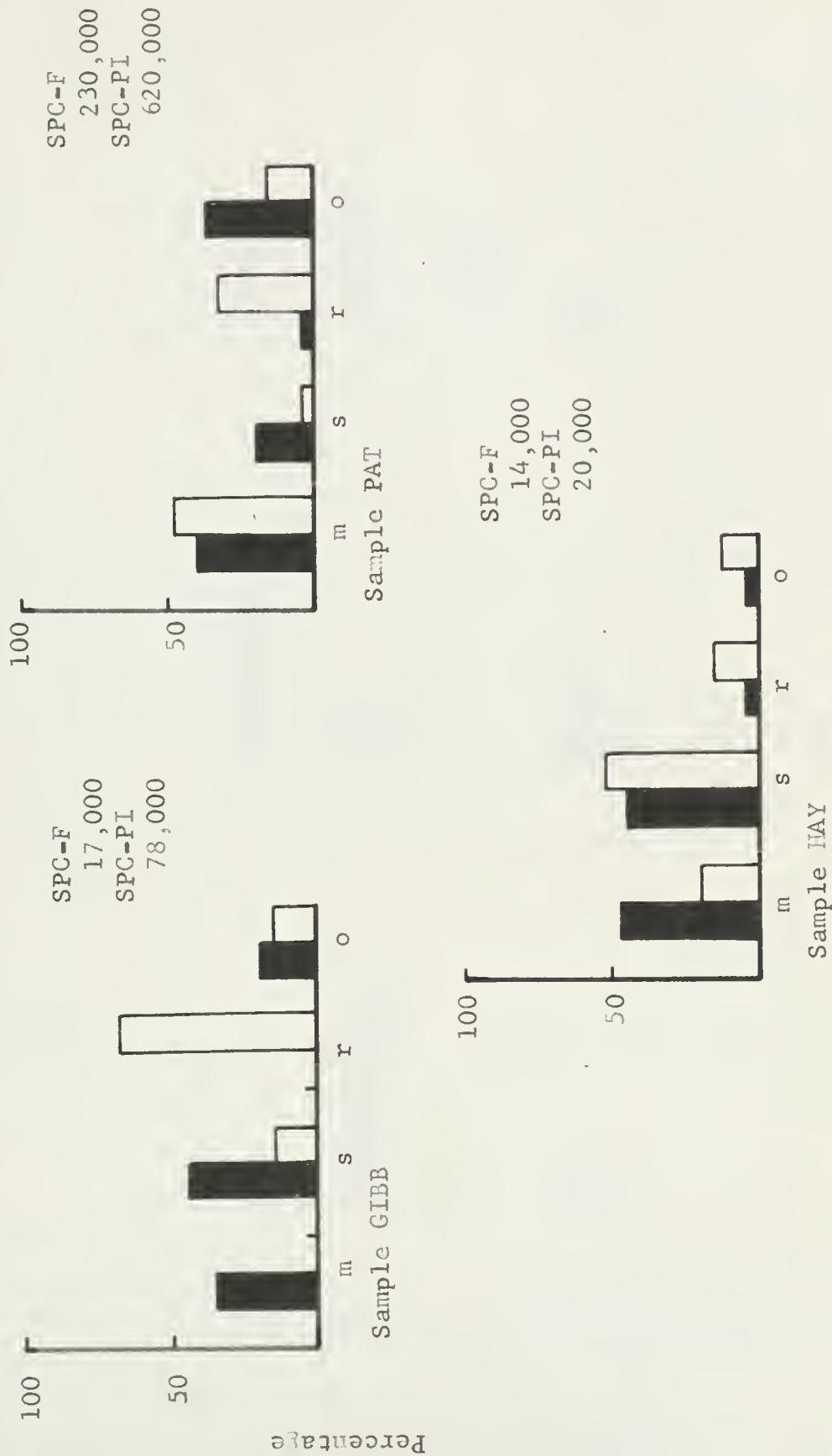


FIGURE 14. (cont.) The relationship between the flora of milk before and after preliminary incubation (P.I.) at 55°F (12.8°C) for 18 hr

Sample

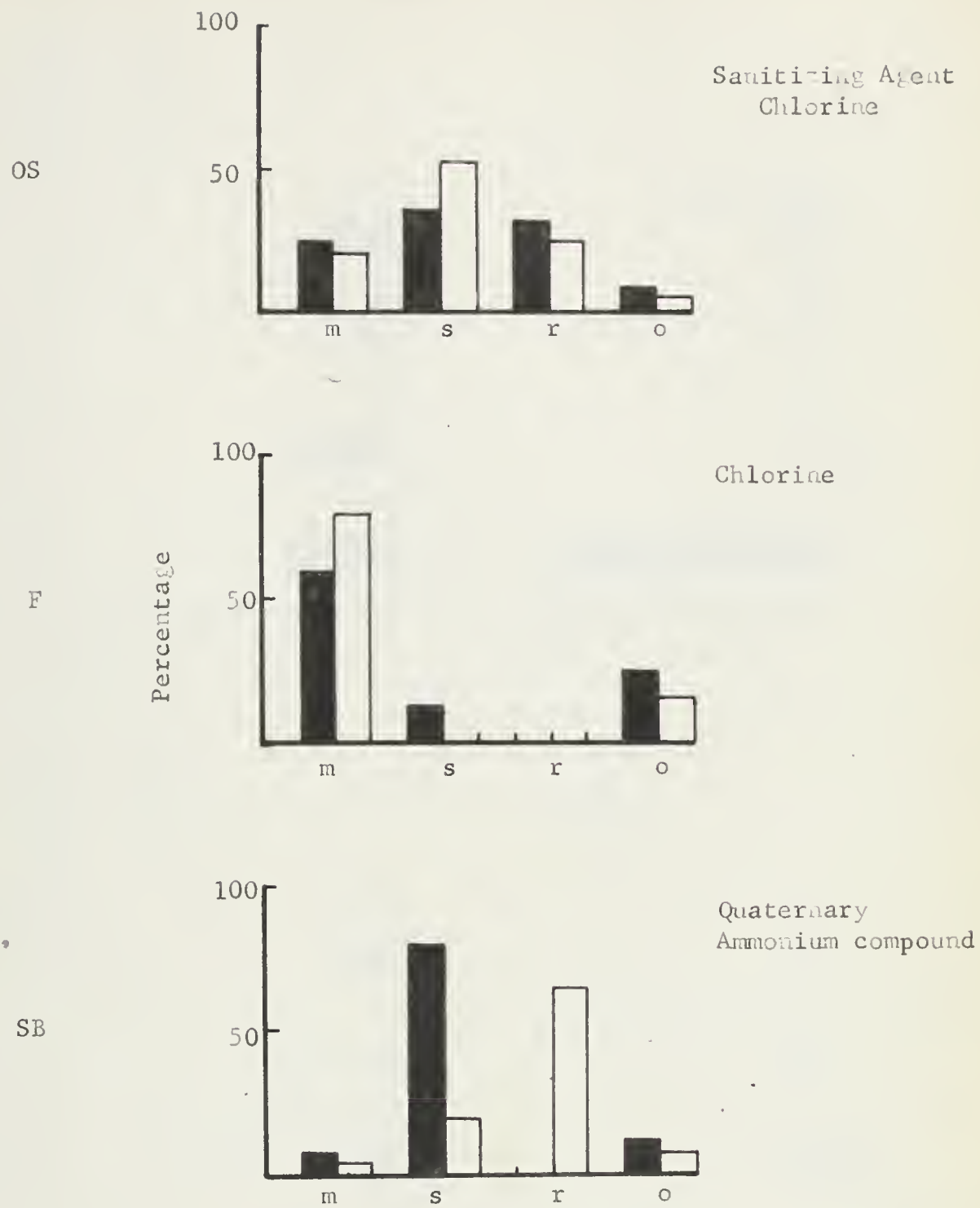


FIGURE 15. The relationship between the flora of **fresh** milk samples and the flora of milking units

■ Fresh milk
□ Milking units

m - micrococci
s - streptococci
r - Gram - ve rods
o - others

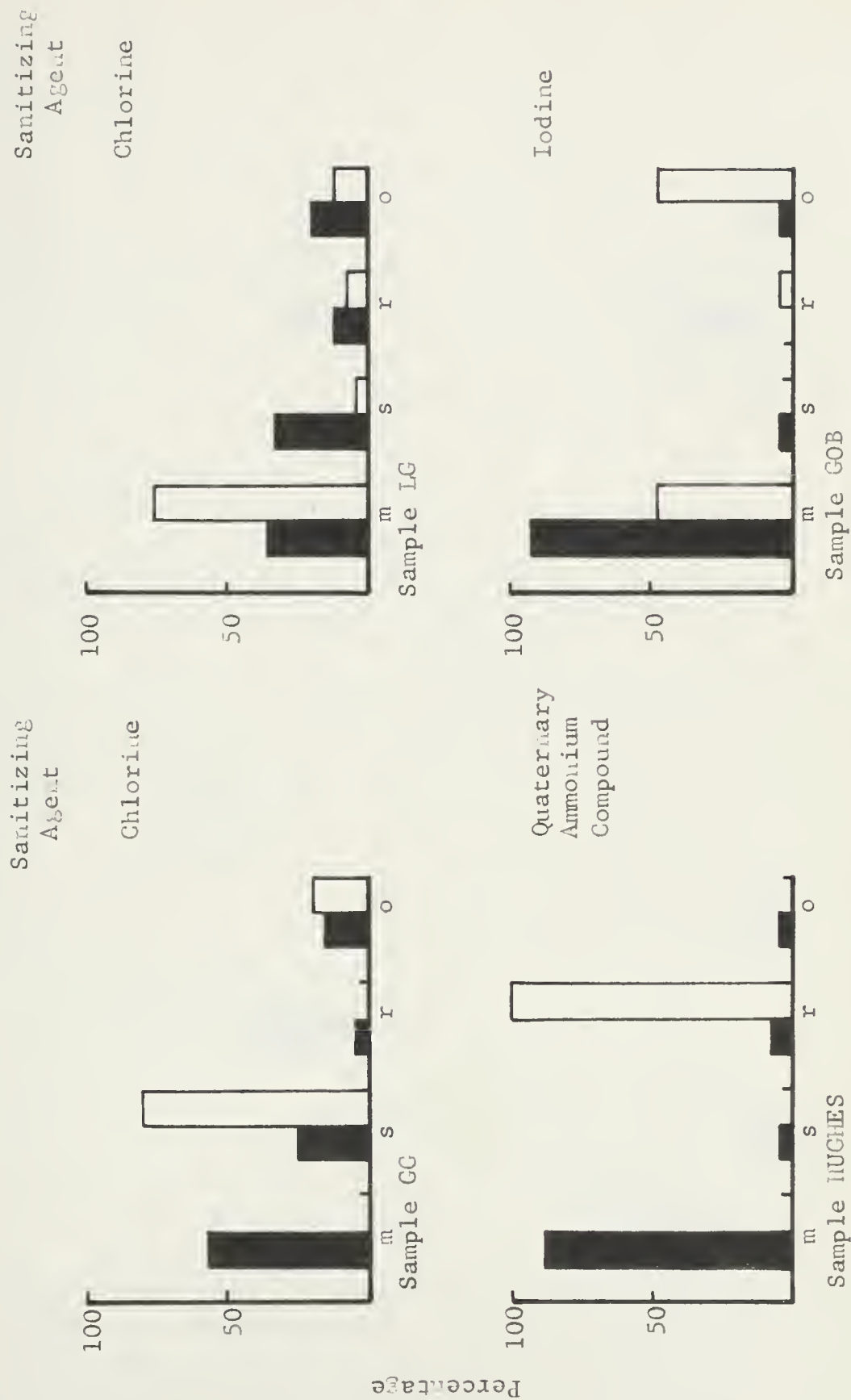


FIGURE 15. (cont.) The relationship between the flora of fresh milk samples and the flora of milking units

- Fresh milk
- Milking units
- m - micrococci
- s - streptococci
- r - Gram - ve rods
- o - others

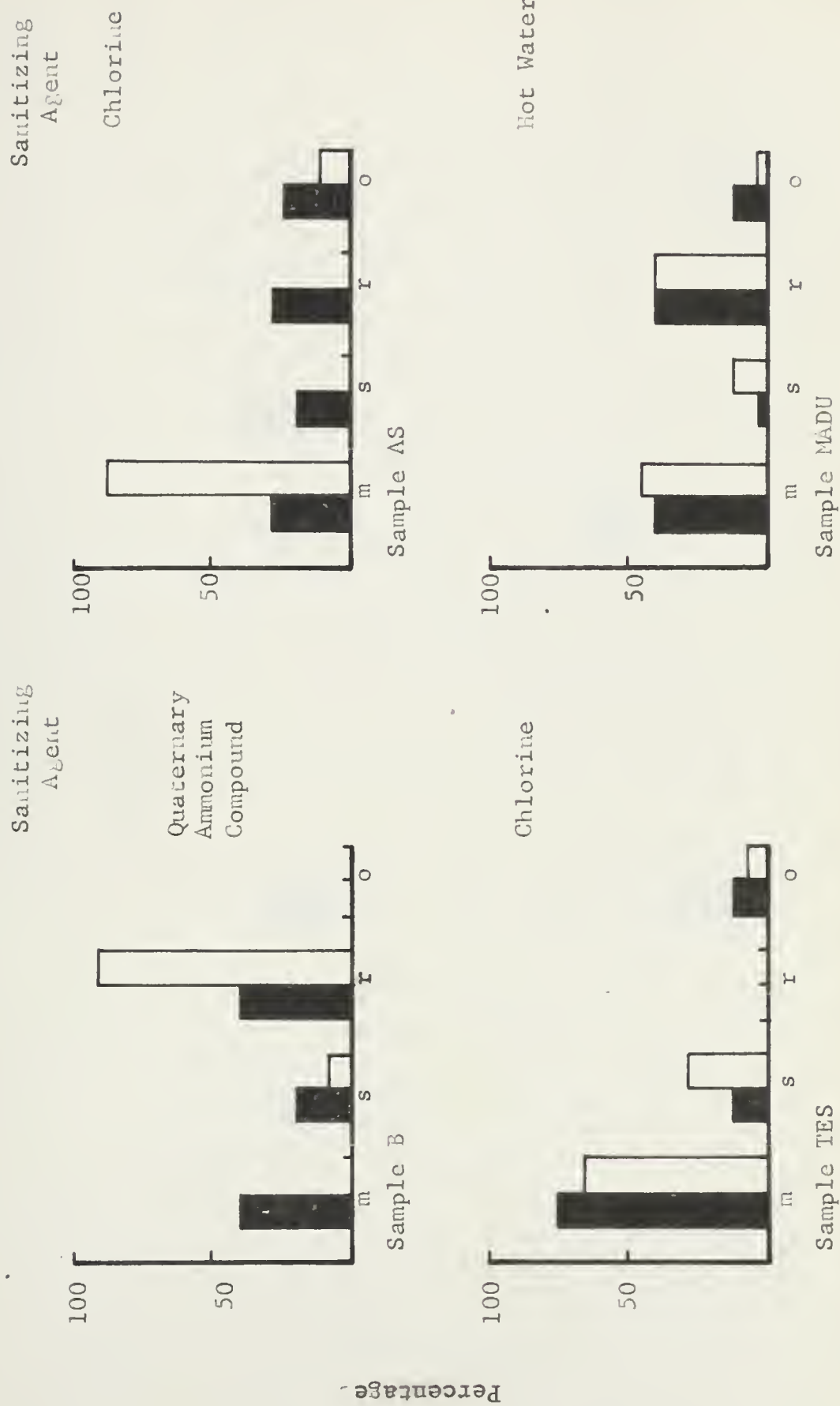


FIGURE 15. The relationship between the flora of fresh milk samples and the flora of milking units

- Fresh milk
- Milking units
- m - micrococci
- s - streptococci
- r - Gram - ve rods
- o - others

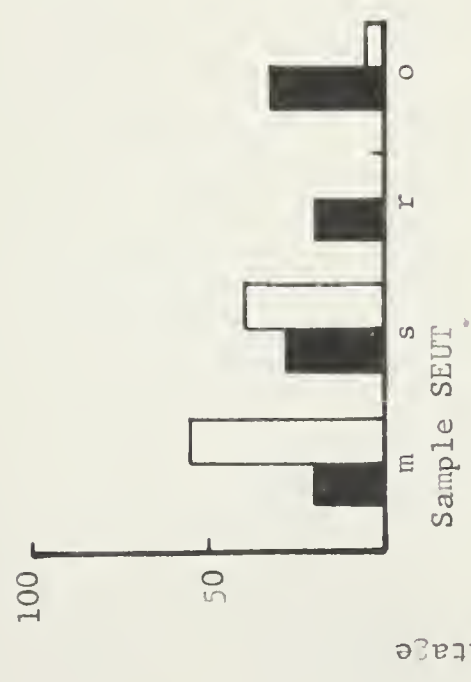
Sanitizing Agent

Chlorine

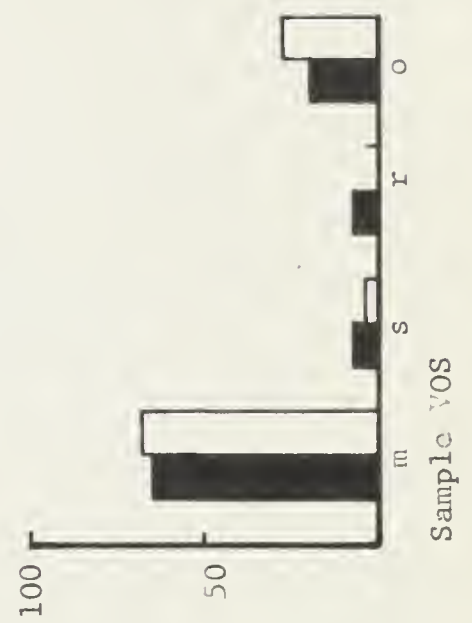


Sanitizing Agent

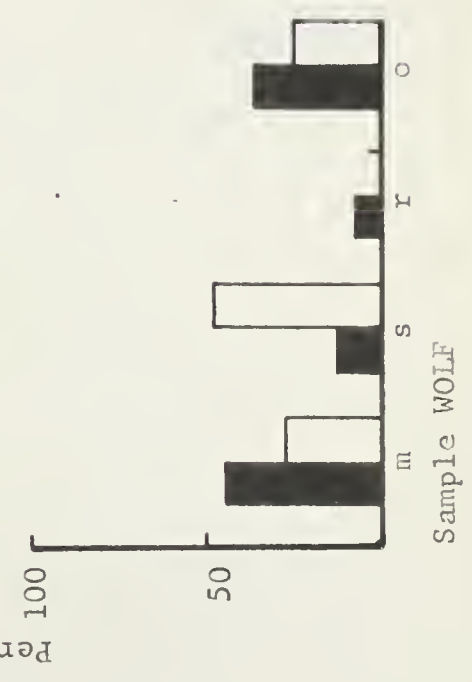
Chlorine



Chlorine



Chlorine



m - micrococci
s - streptococci
r - Gram - ve rods
o - others

■ Fresh milk
□ Milking units

FIGURE 15. (cont.) The relationship between the flora of fresh milk samples and the flora of milking units

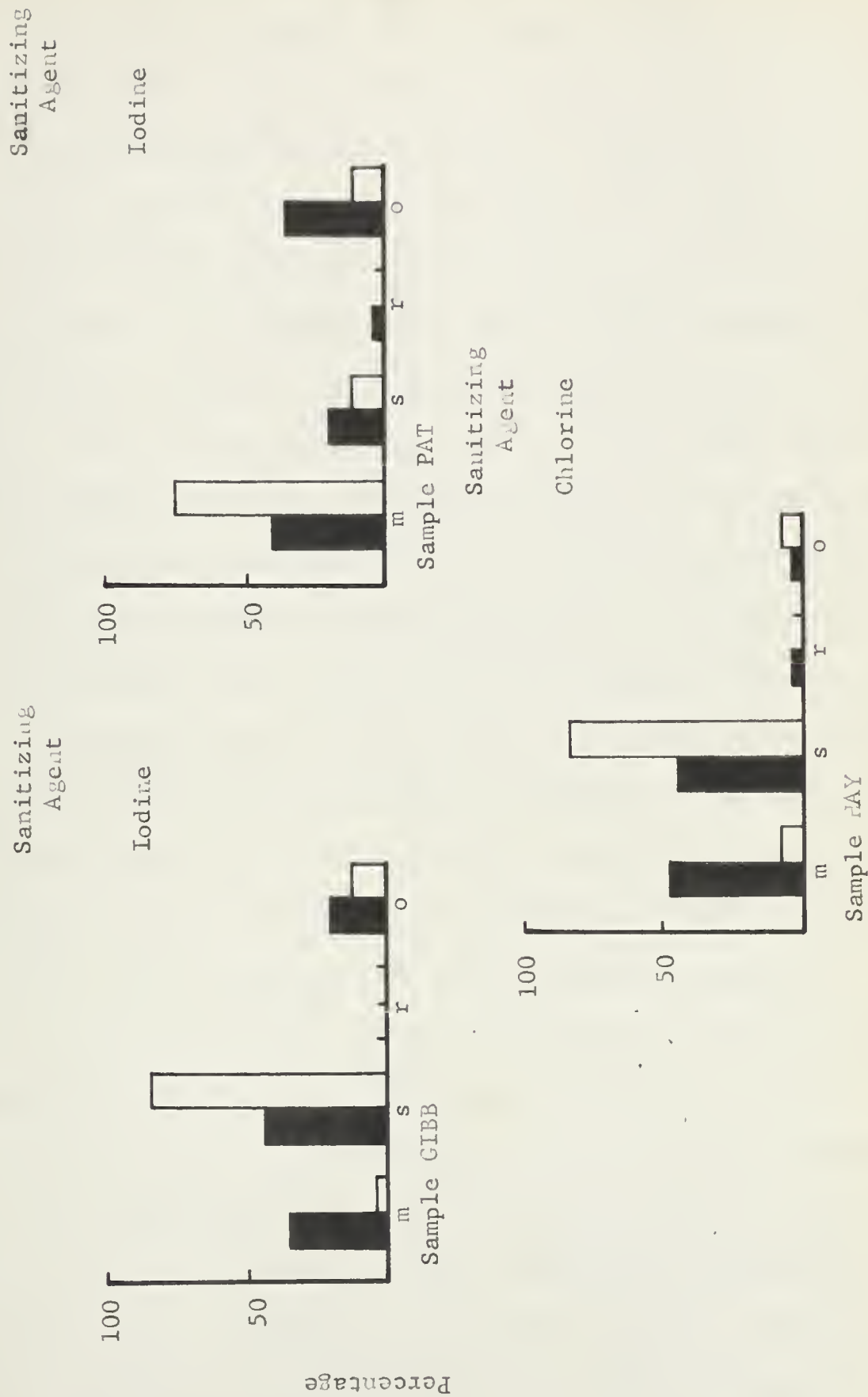


FIGURE 15. (cont.) The relationship between the flora of fresh milk samples and the flora of milking units

standard plate counts of rinses were $>50,000/\text{ft}^2$ streptococci and Gram-negative rods were generally dominant no matter what method of sanitizing was used. A comparison of the data of Thomas et al. and the results obtained in this investigation with respect to numbers and types of bacteria would be of little significance as Thomas et al. did not use a pulsating rinse for rinsing milking units. However, a comparison of the flora in relation to the sanitizing agent can be made with respect to hypochlorite and Q.A.Cs. When hypochlorite (or iodine) was used the flora was dominantly micrococci and when Q.A.Cs. were used (only three cases) Gram-negative rods were dominant. These findings agree with those of Thomas et al. (1963). It is interesting to note that with sample MADU, where hot water of $160-170^{\circ}\text{F}$ ($71-79^{\circ}\text{C}$) was used for sanitizing, a high percentage (40%) of Gram-negative rods was found. As these organisms are not normally considered to be heat resistant it might be that they were protected from the heat in the pores of the rubber inflations during the sanitizing process.

The data on the flora of milking units and the relationship of this to the flora of the fresh milk is difficult to assess. The reason for this is that in none of the samples studied was the pulsating rinse count high enough to contribute appreciably to the plate count of the fresh milk. This is illustrated by the following examples:

On farm S.B. where 4 milking units were in use and a daily milk production of 1600 lbs (720,000 ml) the standard plate count of the pulsating rinse was 5,700,000/milking unit. In 24 hr the milking units were used twice, contributing $4 \times 2 \times 5,700,000$ micro-organisms to the milk - a total of 45,000,000. This is distributed through the milk produced in 24 hr (720,000 ml). Therefore the number of micro-organisms added to each ml of milk = 60 (approx.). However, the plate count on the fresh milk was 9,000.

Similar calculations as above showed that the milking units on other farms contributed approximately 11 micro-organisms/ml of milk to the sample from farm TES, and 320/ml to the sample from farm SEUT.

From these calculations it can be seen that little relationship would be expected between the flora of the milking units and the flora of the fresh milk, the milking units contributing only a small percentage of the bacteria. Allowing even for gross errors in the pulsating rinse technique, it is hard to imagine that the milking units could contribute the required number of organisms to milk to account for all the contamination. Consider, again the following example: Farm SEUT with a daily milk production of 340,000/ml (approx.) yielded a plate count on the fresh milk of 17,000/ml. A generous estimate of the numbers of bacteria in aseptically drawn milk is ca. 5,000/ml. Therefore 12,000 bacteria/ml of milk must have entered the milk from various other sources. The milking units contributed ca. 300/ml. Therefore the net contamination must be ca. 11,700 bacteria/ml. As the daily milk production was 340,000 ml the total number of contaminating bacteria would be $11,700 \times 340,000$ which is 4,000,000,000. The question is where did these bacteria come from if not from the milking units? Similar astronomical figures may be calculated for the other samples.

These large numbers of bacteria could be obtained in several ways:

- (1) from equipment other than the milking units (bulk tank, strainer, etc.);
- (2) during the actual milking procedure due to unsanitary milk handling practices;
- (3) as a result of growth in the bulk tank;
- (4) from the udder.

From the available data it is not possible to draw any conclusions as to which of the above factors is of major importance. However, the following points are worthy of note.

As the milking units are the most difficult pieces of milking equipment to clean and sterilize, it would be expected that they would be

the largest source of bacteria. This implies that the bulk tank, strainers, pails, etc. would add fewer organisms to the milk than the milking units.

Johns (1960) showed that dirty cows, kept in dirty stalls, and milked without udder-washing, had little effect on the bacteriological quality of milk. These results are quite surprising as one would expect dirty teats to be an excellent source of bacteria. Johns, however, stated that the udders were not noticeably soiled. Johns (1962) repeated the above work using badly soiled cows and obtained essentially the same results.

If growth is occurring in the bulk tank (temperature 36 - 40°F) (2.2 - 4.4°C) one would expect this to be reflected in the percentage of Gram-negative rods in the milk. The present results do not indicate such a trend. The presence of psychrophiles other than Gram-negative rods would of course invalidate the above argument. Finally, mastitis and failure to remove the foremilk must be considered as sources of numbers of bacteria. The results of Hucker et al. (1932) and Schalm (1941) indicate that it is not unusual for a cow with mastitis to secrete milk containing 100,000 streptococci/ml. Consider a cow producing 20 lbs of milk a day - i.e. 9,000 ml. Such an animal would add 900 M. bacteria to the milk in the bulk tank. A few such animals in a herd would all but account for the anomalies in any of the earlier calculations. If this were in fact the case one would expect to find large numbers of haemolytic staphylococci or streptococci in the milk. Results with blood agar plates and coagulase tests showed that this was not so. However, Str. dysgalactiae and Str. uberis show little or no haemolytic activity and would not be regarded as pathogenic by their action on blood agar plates.

An alternative explanation of the poor relationship between the

pulsating rinse count and the standard plate count is that bacteria in the pulsating rinse may be in large clumps, and that during the time the milk is held in the bulk tank the clumps disintegrate, either on their own account or because of agitation of the milk in the tank. The effect of this would be reflected in an increased plate count of the milk. This explanation would appear to be more satisfactory than those mentioned above. Contamination of the milk from unhealthy udders, unsanitary milk handling procedures and growth in the bulk tank is probably applicable only in a few instances.

In view of the above discussion, it is suggested that further research on the origin of the bacteria in milk would be both useful and interesting. Such a project would involve a study of the numbers and types of bacteria in aseptically drawn milk, milk as it enters the milking unit, milk as it enters the tank, and milk in the tank before shipment to the dairy plant. At the same time the numbers and types of bacteria on the milking equipment would be studied.

GENERAL DISCUSSION AND CONCLUSIONS

As stated in the General Introduction, the prime reason for undertaking the investigation of the flora of milk, both before and after preliminary incubation (P.I.) at 55°F (12.8°C) for 18 hr was to obtain information that might explain the differences found at four centres in Canada on the effects of P.I. on the standard plate count (S.P.C.) of milk. Thus in Ottawa and Winnipeg the increase in S.P.C. following P.I. was generally greater than the increase at Guelph and Edmonton. It was hoped that this could be explained in terms of the percentages of the different types of micro-organisms in the fresh milk, i.e. samples of milk containing a high percentage of micrococci would show little increase in S.P.C. following P.I., whereas samples containing a high percentage of Gram-negative rods would show a large increase, and results with streptococci would be intermediate between the two. The reasoning was that Gram-negative rods as a whole are the group of micro-organisms most capable of growth at the temperature of P.I. The results in Part II, Sections 3 and 4, show that the increase in S.P.C. following P.I. is not generally related to the percentage of any one particular group of micro-organisms in the fresh milk. In view of this, and the results of Gyllenberg et al. (1959) it is concluded that the increase in S.P.C. following P.I. is related to the growth temperature and the nutritional requirements of the micro-organisms present in the milk.

Of particular interest are the results of the increase in S.P.C. following P.I. reported in Sections 3 and 4. In Section 3, using 39 milk samples which were 48 hr old at the time of testing, the increases in S.P.C. were quite large, 60% of the samples having a ten-fold increase in S.P.C. after P.I. In Section 4, using 18 milk samples which were 24 hr old only 3

(16.7%) increased in S.P.C. after P.I. by a factor of 10 or more. These differences, although not strictly comparable because of the difference in the age of the milk samples, are very marked. The 18 samples studied in Section 4 were from 18 of the 20 farms used in the study of P.I. reported in Part I. As a result of these observations a possible explanation of the differences found at the four centres would be that farms chosen for study yielded micro-organisms with different growth temperature requirements, although the percentages of the various groups of micro-organisms may have in fact been similar. It is suggested that observations on the multiplication of micro-organisms in milk at 55°F (12.8°C) would be valuable.

The results of the study of the flora of raw milk reported here are in general agreement with those reported by Thomas et al. (1962). Although there were differences in technique the trends noted in both investigations are similar, the percentage of micrococci decreasing and the percentage of streptococci and Gram-negative rods increasing with increasing numbers of bacteria in the milk as determined by the S.P.C. In spite of these general trends marked variations were observed. The results are consistent with the theory that milk leaves the healthy udder with a dominant flora of micrococci and as the milk is contaminated the percentage of other types increases.

The results of the flora of milking equipment, although limited to 18 samples, are in agreement with those of Thomas et al. (1963). The differences in the flora as a result of different sanitizing procedures suggests that the quaternary ammonium compounds acted selectively on Gram-positive micro-organisms, whereas the iodine and chlorine disinfectants were less specific in their action.

The investigation on the effects of various types of micro-organisms on the resazurin reduction test (Part II, Section 3) shows that in general

the streptococci and coliforms are active, the micrococci variable, and the Gram-negative rods (other than coliforms) relatively inactive dye reducers. These findings agree with those of Wilson et al. (1935), Hobbs (1939), Thomé (1941) and Garvie and Rowlands (1952b).

The main tests being used in industry for the evaluation of the bacteriological quality of milk are the standard plate count (S.P.C.), the methylene blue and resazurin dye reduction tests. It has been suggested by some workers that the above tests would be more useful following preliminary incubation (P.I.) of the milk. It is necessary to judge each of these tests before and after P.I. on their accuracy as a means of estimating the numbers of bacteria in milk or their ability to indicate the sanitary conditions of milk production.

The S.P.C. is subject to many errors with the result that the accuracy, using duplicate plates and excellent technique, is $\pm 64\%$ of the mean count (Wilson et al. 1935). This does not, however, rule out S.P.C. as a useful control test so long as the error is realized. The most serious disadvantages of the S.P.C. are that it measures clumps of bacteria and only those bacteria which give rise to colonies under the conditions provided; thus absolute numbers are not measured. Whether these factors are important in grading milk is debatable.

The dye reduction tests, being tests of activity, are affected by the types of micro-organisms present as well as their numbers (see above). They are also affected by the presence of leucocytes (e.g. Morris, 1944) and by prolonged refrigeration (see General Introduction). There is no way in which these sources of error can be compensated. The great advantage of the dye reduction tests over the S.P.C. is that they are not affected by the clumping of bacteria. The results of many workers have shown that there

is a good correlation between dye reduction times and bacterial numbers when large numbers of samples are studied (see Figs. 1 and 2). It is concluded from this that the dye reduction tests on large numbers of samples give a good indication of bacterial numbers, however the result on any one sample, because of the factors mentioned above, should be regarded as being of limited value in milk grading. The combined results of a standard plate count and a dye reduction test on a milk sample would appear to give much more information on bacterial numbers than either test alone.

P.I. as suggested by Johns (1958) would appear to remove the effect of inactivation of micro-organisms in the dye reduction tests caused by refrigeration of the milk. P.I. however must introduce another source of error, namely that of an increase in the numbers of bacteria as a result of multiplication during the incubation period. As such a standard plate count or dye reduction test following P.I. could not indicate the numbers of micro-organisms present in the original milk.

The above mentioned tests must now be evaluated as indicators of unsanitary conditions of milk production.

It is generally agreed that aseptically drawn milk from a healthy cow contains a fairly constant number of bacteria, a generous figure being 5000/ml (Hammer and Babel, 1957), although occasionally aseptically drawn milk from healthy cows contains larger numbers (Johns and Hastings, 1938). The subtraction of 5000/ml from the S.P.C. count of a milk sample would thus appear to give a fair estimate of gross contamination. Within certain limits the S.P.C. does not differentiate between psychrophiles, mesophiles and thermophiles, all of which may be contaminants of milk.

The dye reduction tests as indicators of unsanitary conditions of milk production are subject to the same considerations as mentioned above,

namely that they are influenced by the types of bacteria, and numbers of bacteria and leucocytes. As all bacteria in milk (greater than 5000/ml) must be regarded as contaminants, whether they are active or inactive dye reducers, the dye reduction tests may therefore be subject to considerable error in estimating sanitary conditions in milk production. The same argument applies to the dye reduction tests following P.I.

The increase in the S.P.C. following P.I. was suggested by Johns (1958) as a means of assessing the sanitary conditions of milk production. The effect of P.I. on five milk samples from the present studies is given in Table 10.

TABLE 10

The effect of P.I. at 55°F (12.8°C) for 18 hr on
milks of unlike initial bacterial content

Sample No.	S.P.C. (Fresh) (/ml)	S.P.C. (After P.I.) (/ml)	Ratio S.P.C.-P.I.: S.P.C.-Fresh
173	7,000	14,000	2.00
123	7,200	110,000	15.30
V A	7,000	800,000	114.30
190	110,000	130,000	1.2
184	92,000	220,000	2.4

Workers in favour of P.I. might interpret the above results as follows: Sample 173 contained bacteria derived mainly from the udder and hence little growth occurred during P.I.; sample 123 contained a fair percentage of saprophytes as a result of unsanitary methods of milk production and hence some multiplication occurred during P.I.; sample V A

contained a large percentage of saprophytes as a result of unsanitary milk production and hence a great deal of multiplication occurred during P.I. The main argument against this interpretation is that milk from a healthy cow leaves the udder with a bacterial content of $< 5000/\text{ml}$. Thus if $5000/\text{ml}$ is subtracted from each of the standard plate counts before P.I. in the above table the figures of 2000, 2200, and 2000 bacteria/ml are obtained; these figures representing bacteria which have gained access to the milk from sources other than healthy cows. Should these organisms grow well at the temperature of P.I. a result such as obtained with sample V.A. would be expected; if they do not than a result such as obtained with sample 173 would be expected.

Consider also the results of samples 190 and 184. The subtraction of 5000 bacteria/ml from the standard plate counts before P.I. gives the figures of 105,000 and 87,000 bacteria/ml. The effect of P.I. in both cases was to bring about only a small increase in bacterial numbers. Even though gross contamination was evident in each case little multiplication took place, indicating that in each sample only a few bacteria were capable of growth at the temperature of P.I. The data cited in Table 10 are only a few examples; many similar examples are present in the results in Parts I and II.

It is concluded that the increase in numbers of bacteria following P.I. of milk samples merely reflects the presence of certain bacteria in the fresh milk with the ability to multiply at the temperature of P.I.

The use of the increase in bacterial numbers following P.I. as an index of unsanitary conditions of milk production presupposes that the bacteria gaining entry to the milk through such conditions are capable of growth at the temperature of P.I. The results of the analysis of the flora of milking equipment do not support such a supposition, assuming that micro-

cocci and streptococci, in general, are capable of only limited growth at 55°F. There is no reason why large numbers of bacteria derived from the udder should not be regarded as undesirable contaminants of milk. The effect of P.I. on these bacteria would be expected to be small. On the basis of this P.I. - unlike the S.P.C. - differentiates between psychrophilic, mesophilic and thermophilic contaminants favoring psychrophiles and discriminating against mesophiles and thermophiles. Under certain circumstances this might be useful. Thus when milk is collected from the farms it is taken to the dairy where it is often stored overnight at a temperature below 40°F (4.4°C), before being pasteurized the following day. During this period of storage the presence of psychrophiles is undesirable as they are the micro-organism most likely to multiply. In view of this the detection of micro-organisms in milk capable of growth at low temperatures, as evidenced by a standard plate count after P.I. would appear to be useful. The non-acceptance of milk from farms showing large increases in bacterial numbers following P.I. could safeguard the commercial dairy plant from defects as a result of overnight storage of raw milk.

From the discussion presented above it is suggested that when performed by skilled personnel the S.P.C. is a reliable means of assessing both the numbers of bacteria in milk and the conditions under which the milk was produced. The limits of error would not seriously affect the grading of market milk. The results of dye reduction tests, although of limited value on individual milk samples, are useful when frequent tests are made on individual supplies. P.I. as a means of overcoming the effects of prolonged refrigeration on dye reduction times would not appear to be the best solution to this problem as there is no way of predicting which group or groups of micro-organisms will be favourably affected by P.I. and whether such multiplication will have any effect on the dye

reduction test. Another possibility of reactivating bacteria in refrigerated milk is the use of some shorter time and higher temperature than suggested by Johns (1958). Such time would need to be less than the shortest generation time of any bacteria present in the milk (say 10 min) to avoid bacterial multiplication during the incubation.

A routine test of importance, although not covered in this work, is the laboratory pasteurized count. The results of this test indicate the number of organisms in milk which survive pasteurization and may, if present in sufficient numbers in raw milk, give rise to large numbers in pasteurized milk.

The question should now be asked "What should be considered as reasonable standards for the bacteriological quality of milk?" The purist would require that milk should contain a maximum of say 10,000 bacteria/ml, be free from pathogenic bacteria, and contain few bacteria that can survive pasteurization. The figure of 10,000 bacteria/ml may be rather low, however Clegg (1955) found that milk producers could easily obtain a standard of $< 10,000/\text{ml}$. If such a high standard can be achieved on any one farm it must be possible on all farms. As milk of a low plate count can be produced under visually dirty conditions the purist would also require that the conditions under which milk is produced are aesthetically acceptable. This can only be completely achieved by the introduction and enforcement of suitable regulations, though the sediment test goes a long way towards this.

Opposed to the purist are those who contend that so long as milk has no organoleptic defects, is free from pathogenic organisms, and does not contain excessive numbers of thermoduric organisms, it does not matter how many bacteria are present in the milk or under what conditions the milk is produced, especially if the milk is to be pasteurized.

Most people however would be inclined to agree with the purist, feeling that it is aesthetically repulsive to think of milk being produced under unsanitary conditions. Only by setting rigid standards and enforcing them can the desired results be obtained.

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APPENDIX A

Results of the analysis of the flora, leucocyte count and standard plate count of 39 milk samples

Sample number	Micrococci	Streptococci	Asporogenous Gram +ve rods	Gram -ve rods	Bacilli	Coliforms	Unclassified	Resazurin reduced time (hr)	Leucocytes x 10 ³ /ml	Standard plate count x 10 ³ /ml	Ratio SPC-P.I.: SPC-F
VA F	28	-	8	56	-	-	8	10	350	7	114.3
P.I.	-	-	-	100	-	-	-	9		800	
8 F	32	32	-	20	-	-	16	9	530	17	3.9
P.I.	16	4	-	24	-	44	12	6		66	
11 F	8	16	-	72	-	-	4	7	930	370	11.9
P.I.	-	-	-	100	-	-	-	3		4,400	
14 F	32	44	-	12	-	-	12	8	750	24	3.7
P.I.	16	28	-	52	4	-	-	6		90	
16 F	56	-	16	16	-	8	4	10	200	6.5	146.1
P.I.	12	4	4	60	-	20	-	7		950	
24 F	32	16	8	32	8	-	4	10	350	5.3	7.5
P.I.	-	8	-	92	-	-	-	7		40	
25 F	-	12	-	88	-	-	-	6	300	1,400	10
P.I.	-	-	-	100	-	-	-	3		14,000	
33 F	52	24	12	4	-	-	8	9	440	11	7.3
P.I.	24	20	20	20	-	8	8	7		80	

F = fresh

P.I. = preliminary incubation

L.A. = laboratory accident

S.P.C. = standard plate count

APPENDIX A (continued)

Results of the analysis of the flora, leucocyte count and standard plate count of 39 milk samples

Sample number	Micrococci	Streptococci	Asporogenous Gram +ve rods	Gram -ve rods	Bacilli	Coliforms	Unclassified	Resazurin reduction time (hr)	Leucocytes x10 ³ /ml	Standard plate count x10 ³ /ml	Ratio SPC-P.I.: SPC-F
35 F	8	-	-	92	-	-	-	3	760	550	40
P.I.	8	-	-	92	-	-	-	2		22,000	
39 F	4	-	4	76	-	16	-	8	360	25	128
P.I.	-	-	-	80	-	20	-	5		3,200	
43 F	4	-	28	48	-	-	20	10	200	22	59.1
P.I.	4	-	-	88	-	-	8	8		1,300	
61 F	48	-	32	20	-	-	-	8	480	70	92.9
P.I.	-	-	-	100	-	-	-	6		6,500	
65 F	36	24	-	32	4	-	4	8	L.A.	7.3	54.8
P.I.	-	-	-	88	4	-	8	6		400	
67 F	48	8	-	28	4	-	12	10	240	2.4	3.7
P.I.	24	8	-	52	-	4	12	9		9	
67aF	20	4	-	56	-	-	20	8	560	14	12.8
P.I.	-	-	-	100	-	-	-	6		180	
68 F	32	20	12	20	-	-	16	8	700	54	53.7
P.I.	8	-	4	84	-	-	4	4		2,900	
72 F	8	4	24	48	4	-	12	10	L.A.	8.6	1.4
P.I.	28	24	12	28	8	-	-	9		12	

F = fresh

P.I. = preliminary incubation

L.A. = laboratory accident

S.P.C. = standard plate count

APPENDIX A (continued)

Results of the analysis of the flora, leucocyte count and standard plate count of 39 milk samples

Sample number	Micrococci	Streptococci	Asporogenous Gram +ve rods	Gram -ve rods	Bacilli	Coliforms	Unclassified	Resazurin reduction time (hr)	Leucocytes x10 ³ /ml	Standard plate count x10 ³ /ml	Ratio SPC-P.I.: SPC-F
75 F	24	40	4	28	-	-	4	8	800	25	120
P.I.	8	12	4	72	-	-	4	8		300	
85 F	36	12	8	24	-	-	20	10	480	18	11.1
P.I.	4	4	-	84	4	-	4	8		200	
92 F	48	20	-	12	8	-	12	8	400	22	29.5
P.I.	-	4	-	92	-	-	4	6		650	
105 F	32	16	12	36	-	-	4	10	520	14	5.0
P.I.	20	-	-	76	-	-	4	9		70	
117 F	24	12	-	44	-	-	20	10	L.A.	5.5	32.7
P.I.	-	-	-	100	-	-	-	8		180	
120 F	36	56	4	-	-	-	4	6	220	130	1.8
P.I.	8	84	-	-	-	-	8	4		240	
123 F	16	28	12	40	-	-	4	10	240	7.2	15.3
P.I.	44	32	8	16	-	-	-	7		110	
143 F	8	4	-	84	-	-	4	9	L.A.	53	43.4
P.I.	-	-	-	100	-	-	-	6		2,300	

F = fresh

P.I. = preliminary incubation

L.A. = laboratory accident

S.P.C. = standard plant count

APPENDIX A (continued)

Results of the analysis of the flora, leucocyte count and standard plate count of 39 milk samples

Sample number	Micrococci	Streptococci	Asporogenous Gram +ve rods	Gram -ve rods	Bacilli	Coliforms	Unclassified	Resazurin reduction time (hr)	Leucocyte $\times 10^3/\text{ml}$	Standard plate count $\times 10^3/\text{ml}$	Ratio SPC-P.I.: SPC-F
144 F	32	8	32	28	-	-	-	7	610	190	3.2
P.I.	56	4	8	28	-	-	4	5		600	
145 F	16	4	12	52	-	-	16	6	440	280	67.9
P.I.	8	-	4	68	4	-	16	3		19,000	
154 F	40	32	-	12	-	-	16	10	620	12	31.7
P.I.	-	-	-	100	-	-	-	8		380	
155 F	64	4	16	-	4	-	12	10	880	8.3	3.6
P.I.	32	-	16	52	-	-	-	9		30	
160 F	48	28	4	8	-	-	12	9	600	11	10
P.I.	-	4	28	64	-	-	4	8		110	
163 F	12	12	-	52	-	4	20	8	960	170	10
P.I.	4	8	4	80	-	-	4	4		1,700	
165 F	12	24	-	28	4	16	16	9	810	55	38.2
P.I.	-	-	-	100	-	-	-	7		2,100	
173 F	60	8	8	16	4	-	4	10	400	7	2.0
P.I.	24	-	-	76	-	-	-	9		14	

F. = fresh

P.I. = preliminary incubation

L.A. = laboratory accident

S.P.C. = standard plate count

APPENDIX A (continued)

Results of the analysis of the flora, leucocyte count and standard plate count of 39 milk samples

Sample number	Micrococci	Streptococci	Asporogenous Gram +ve rods	Gram -ve rods	Bacilli	Coliforms	Unclassified	Resazurin reduction time (hr)	Leucocytes x 10 ³ /ml	Standard plate count x10 ³ /ml	Ratio SPC-P.I. SPC-F
184 F	24	56	8	8	-	-	4	7	840	92	2.4
P.I.	20	16	8	48	-	-	8	4		220	
188 F	70	-	-	30	-	-	-	9	760	2.8	4.3
P.I.	20	-	-	80	-	-	-	8		12	
189 F	20	40	4	24	-	12	-	7	550	29	58.6
P.I.	12	16	-	48	-	20	4	4		1,700	
190 F	56	28	8	-	-	-	8	8	1300	110	1.2
P.I.	44	32	24	-	-	-	-	7		130	
194 F	-	-	20	52	-	-	28	6	410	390	66.7
P.I.	-	-	4	72	-	-	24	2		26,000	
200 F	80	-	8	4	4	-	4	9	400	1.8	7.2
P.I.	76	-	-	24	-	-	-	7		13	

F = fresh

P.I. = preliminary incubation

L.A. = laboratory accident

S.P.C. = standard plate count

APPENDIX B

Results of the analysis of the flora and standard plate counts
of 18 milk samples and 18 samples of rinses of milking units

Sample	Micrococci	Streptococci	Asporogenous Gram +ve rods	Gram -ve rods	Bacilli	Coliforms	Unclassified	Standard plate* count/ml x10 ³	Ratio SPC-P.I.: SPC-F	Sanitizing Agent
OS.F	24	36	8	32	-	-	-	12	3.3	
P.I.	12	28	-	60	-	-	-	40		
R	20	52	-	24	-	-	4	32,000		Chlorine
F.F.	60	16	8	-	-	-	16	2.7	3.3	
P.I.	40	40	8	-	-	-	12	9.0		
R	80	-	4	-	4	-	12	90.0		Chlorine
SB.F	8	80	-	-	-	-	12	9.0	1.8	
P.I.	44	48	-	-	-	-	8	16.0		
R	4	20	-	68	-	-	8	5,700		Q.A.C.
B.F.	40	20	-	40	-	-	-	31	138.9	
P.I.	-	-	-	100	-	-	-	4,300		
R	-	8	-	92	-	-	-	14,000		Q.A.C.
AS.F	28	20	4	28	-	-	20	25	2.0	
P.I.	12	12	8	52	-	-	16	50		
R	88	-	-	-	-	-	12	58		Chlorine
TES F	76	12	4	-	-	-	8	2.8	1.2	
P.I.	60	12	8	16	-	-	4	3.3		
R	66	28	-	-	-	-	8	540		Chlorine

F = fresh milk
P.I. = milk after P.I.
R = rinse
S.P.C. = standard plate count

*results of S.P.C. for rinses are
reported as number/milking unit

Q.A.C. = quaternary ammonium compound

APPENDIX B (continued)

Results of the analysis of the flora and standard plate counts
of 18 milk samples and 18 samples of rinses of milking units

Sample	Micrococci	Streptococci	Asporogenous Gram +ve rods	Gram -ve rods	Bacilli	Coliform	Unclassified	Standard plate* count/ml x10 ³	Ratio SPC-P.I.: SPC-F	Sanitizing Agent
Madu F	40	4	8	40	-	-	4	34	67.7	Hot water
P.I.	-	-	-	100	-	-	-	2,300		
R	44	12	-	40	-	-	4	65,000		
Seut F	20	28	-	20	-	24	8	17	1.9	Chlorine
P.I.	28	12	4	44	-	8	4	32		
R	56	40	-	-	-	-	4	18,000		
Ten F	36	44	8	12	-	-	-	8.2	48.8	Chlorine
P.I.	-	4	-	96	-	-	-	400		
R	16	-	16	52	-	-	16	650		
Wolf F	44	12	24	8	-	4	8	16	1.6	Chlorine
P.I.	32	4	8	52	-	-	4	26		
R	28	48	4	-	4	-	16	420		
Vos F	64	8	-	8	-	-	20	12	1.3	Chlorine
P.I.	56	16	8	4	-	-	16	16		
R	68	4	4	-	4	-	20	760		
GG F	56	24	8	4	-	-	8	15	8.0	Chlorine
P.I.	12	44	-	36	-	8	-	120		
R	-	80	4	-	-	-	16	110		

F = fresh milk
P.I. = milk after P.I.
R = rinse
S.P.C. = standard plate count

*results of S.P.C. for rinses are
reported as number/milking unit

Q.A.C. = quaternary ammonium compound

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